(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2001 (01.11.2001)

PCT

(10) International Publication Number
WO 01/81573 A1

(51) International Patent Classification[7]: C12N 15/12, C07K 14/705, C12Q 1/68, G01N 33/52, 33/50

(21) International Application Number: PCT/EP01/04283

(22) International Filing Date: 14 April 2001 (14.04.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

| | | |
|---|---|---|
| 00108858.2 | 26 April 2000 (26.04.2000) | EP |
| 00116589.3 | 1 August 2000 (01.08.2000) | EP |

(71) Applicant: AVENTIS PHARMA DEUTSCHLAND GMBH [DE/DE]; Brüningstrasse 50, 65929 Frankfurt (DE).

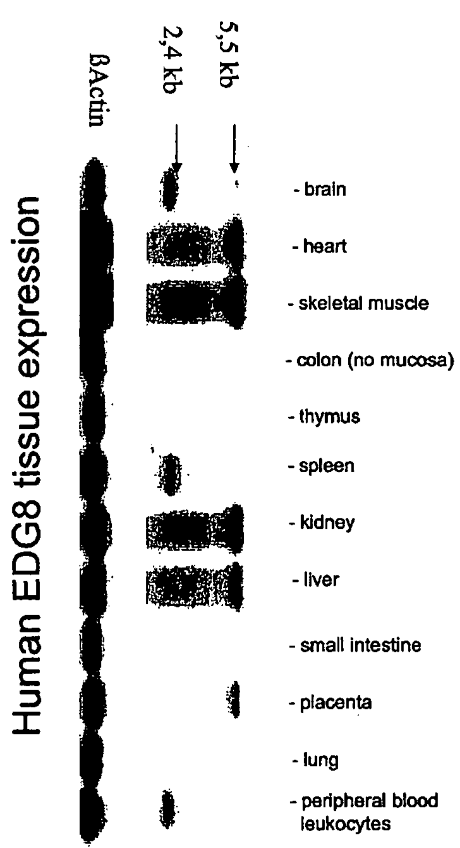
(72) Inventors: KOSTENIS, Evi; Deutschherrenufer 35, 60594 Frankfurt am Main (DE). GASSENHUBER, Johann; Emanuel Geibel Strasse 8, 65185 Wiesbaden (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

*[Continued on next page]*

# (54) Title: EDG8 RECEPTOR, ITS PREPARATION AND USE




**(57) Abstract:** The present invention relates to newly identified EGD8 receptors, polynucleotides encoding this receptor, polypeptides encoded by such polynucleotides, the preparation and the use of such polynucleotides and polypeptides.

WO 01/81573 A1

**Published:**
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

EDG8 receptor, its preparation and use

The present invention relates to newly identified EGD8 receptors, polynucleotides encoding this receptor, polypeptides encoded by such polynucleotides, the preparation and the use of such polynucleotides and polypeptides.

In an effort to identify new G-protein coupled receptors of the EDG (endothelial differentiation gene)-family a novel member of the EDG-family of G-protein coupled receptors, Human EDG8, was identified. The full-length clone was isolated and studies on chromosomal mapping, tissue expression and identification as a functional cellular receptor for sphingosine 1-phosphate were performed. Taken together, the data provide compelling evidence that EDG8 is the fifth receptor for sphingosine 1-phosphate, exclusively expressed in peripheral tissues, its presence in endothelial cells being responsible for the broad tissue distribution.

The lysolipid phosphate mediators lysophosphatidic acid (LPA) and sphingosin 1-phosphate (S1P) have attracted increasing attention as modulators of a variety of important biological functions (Moolenaar et al., 1997; Morris, 1999; Lynch and Im, 1999) and their list of biological activities is continuously growing.
Among the biological responses to LPA is platelet aggregation (Jalink et al., 1994; Siess et al., 1999; Gueguen et al., 1999), smooth muscle contraction (Tokumura et al., 1980), in vivo vasoactive effects (Tokumura et al., 1995), chemotaxis (Jalink et al., 1993), expression of adhesion molecules (Lee et al., 1998b; Rizza et al., 1999), increased tight junction permeability of endothelial cells (Schulze et al., 1997), induction of stress fibers (Gohla et al., 1998) and many others (for review see Moolenaar et al., 1997). The biochemical signalling events that mediate the cellular effects of LPA include stimulation of phospholipases, mobilization of intracellular $Ca^{2+}$, inhibition of adenylyl cyclase, activation of phosphatidylinositol 3-kinase, activation of the Ras-Raf-MAP kinase cascade and stimulation of Rho-GTPases (Moolenaar et al., 1997).
S1P, in particular, is implicated in cell proliferation, modulation of cell motility (reviewed in Hla et al., 1999) induction/suppression of apoptosis (Hisano et al., 1999; Xia et al.,

1999), angiogenesis (Lee et al., 1999), tumor invasiveness (Sadahira et al., 1992), platelet activation (Gueguen et al., 1999) and neurite retraction (Postma et al., 1996). Cellular signalling by S1P involves activation of PLCß and subsequent intracellular $Ca^{2+}$ release (van Koppen et al., 1996; Meyer zu Heringdorf et al., 1997; Yatomi et al., 1997a; Noh et al., 1998; Ancellin and Hla, 1999), activation of MAP-kinases (Wu et al., 1995; Lee et al., 1996; An et al., 2000), activation of inward rectifying $K^+$-channels (van Koppen et al., 1996; Bünemann et al., 1996) and inhibition and/or activation of adenylyl cyclase (Lee et al.,.1996).

Both, LPA and S1P are recognized to signal cells through a set of G-protein coupled receptors (GPCRs) known as EDG (endothelial differentiation gene)-receptors. The EDG-family of GPCRs currently comprises seven human members (EDG1-7) that fall into two major groups depending on their preference for the activating lipid-ligand: EDG1, 3, 5 and 6 preferentially interact with S1P (Yatomi et al., 1997b; Lee et al., 1998a,b; Ancellin and Hla, 1999; Yamazaki et al., 2000; Van Brocklyn et al., 2000), EDG2, 4 and 7 preferentially interact with LPA (An et al., 1998; Im et al., 2000 ).

The assignment of specific biological functions to certain receptor subtypes is hampered by the fact that EDG receptors are expressed in an overlapping fashion (Rizza et al., 1999; Lee et al., 1999), they activate multiple and in part redundant signal transduction pathways (Lee et al., 1996; Ancellin and Hla, 1999; Kon et al., 1999; An et al., 2000), the selectivity for their activating ligands is not absolute (Lee et al., 1998b), and medicinal chemistry is only poorly developed in that specific antagonists for dissecting the pharmacology of the individual subtypes are not available yet.

An important step to shed more light on the biological role of the individual receptor subtypes would be to identify the complete set of receptors that respond to the phospholipid mediators S1P and LPA.

The present invention relates to newly identified EGD8 receptors, polynucleotides encoding this receptor, polypeptides encoded by such polynucleotides the preparation and the use of thereof.

The present invention relates to an isolated polynucleotide comprising a nucleotide sequence that has at least 90 % identity, preferably 95 % or more, most preferably 98 % identity to a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or the corresponding fragment thereof; or a nucleotide sequence complementary to said nucleotide sequence.

Preferably, the polynucleotide is DNA or RNA. The nucleotide sequence of the polynucleotide is at least 90 % identical to that contained in SEQ ID NO. 1.; preferably 95 % or more, most preferred 98 % or more identical to SEQ ID NO. 1. In another embodiment, the polynucleotide has the nucleotide sequence SEQ ID NO. 1. In another embodiment, the polynucleotide encodes the polypeptide of SEQ ID NO. 2 or a fragment thereof. In a special embodiment, the polynucleotide is an allel of SEQ ID NO. 1. Preferably, the polynucleotide has the same essential properties and/or biological functionality as human EDG8.
One characteristic functionality is that the polynucleotid encodes for a S1P receptor; it responds to S1P and optionally also to related phospholipids like DMS 1P or LPA.

Another aspect of the invention relates to an expression system for the expression of EDG8. The EDG8 DNA or RNA molecule comprising an expression system wherein said expression system is capable of producing a polypeptide or a fragment thereof having at least 90 % identity, preferably 95 % or more, most preferred 98 % or more identity with a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or said fragment when said expression system is present in a compatible host cell. Preferably, the expression system is a vector.
The invention relates to a host cell comprising the expression system.

In another aspect, the invention relates to a process for producing an EDG8 polypeptide or a fragment thereof wherein a host cell comprising the expression system is cultured under conditions sufficient for the production of said polypeptide or fragment thereof.
Preferably, the said polypeptide or fragment thereof is expressed at the surface of said cell.

The invention relates also to cells produced by this process.

The process preferably further includes recovering the polypeptide or fragment thereof from the culture.

In another aspect, the invention relates to a process for producing a cell which produces an EDG8 polypeptide or a fragment thereof comprising transforming or transfecting a host cell with the expression system such that the host cell, under appropriate culture conditions, produces an EDG8 polypeptide or a fragment thereof.

In particular, the invention relates to an EDG8 polypeptide or a fragment thereof comprising an amino acid sequence which has at least 90 %, preferably 95 %, most preferred 98 % or more identity to the amino acid sequence SEQ ID NO. 2 or to a part of SEQ ID NO. 2. In particular the invention relates to an EDG8 polypeptide or a fragment thereof having amino acid sequence SEQ ID NO. 2 or a part thereof. In particular, the invention relates to an polypeptide encoded by SEQ ID NO. 1 or encoded by a polynucleotide that has at least 90 %, preferably 95 %, most preferred 98 % or more identity with SEQ ID NO. 1; preferably, such polypeptid has almost the same properties as human EDG 8; e.g. the same biological functionality. One characteristic functionality of human EDG8 is that the polypeptid is a S1P receptor; it responds to S1P and optionally to related phospholipids like DMS1P or LPA.

Further, the invention relates to a process for diagnosing a disease or a susceptibility to a disease related to expression or acitivity of EDG8 polypeptide comprising:

a) determining the presence or absence of mutation in the nucleotide sequence encoding said EDG8 polypeptide in the genome of said subject; and/or

b) analyzing for the presence or amount of the EDG8 polypeptide expression in a sample derived from said subject.

In addition, the invention relates to a method for identifying compounds which bind to EDG8 polypeptide comprising:

a) contacting a cell comprising the expression system or a part of such a cell with a candidate compound; and
b) assessing the ability of said candidate compound to bind to said cells.

Preferably, the method for identifying compounds further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an agonist.

In another embodiment of the invention, the method for identifying compounds further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an antagonist.

The invention also relates to an agonist or antagonist identified by such methods.

In another special embodiment of the invention, the method further includes contacting said cell with a known agonist for said EDG8 polypeptide; and determining whether the signal generated by said agonist is diminished in the presence of said candidate compound, wherein a candidate compound which effects a diminution in said signal is identified as an antagonist for said EDG8 polypeptide. The known agonist is for example S1P, LPA and/or DHS1P. The invention also relates to an antagonist identified by the method.

The invention in addition, relates to a method of preparing a pharmaceutical composition comprising

a) identifying a compound which is an agonist or an antagonist of EDG8,
b) preparing the compound, and
c) optionally mixing the compound with suitable additives.

The invention also relates to a pharmaceutical compound prepared by such a process.

The invention relates to a pharmaceutical, comprising as active ingredient for example such identified compound, an EDG8 polypeptid or a polynucleotide encoding for EDG8 or a part thereof.

In particular, the invention relates to a pharmaceutical, that can be used for the prevention and/or treatment of diseases associated with EDG8/S1P signal transduction, for example diseases associated with endothelial dysfunction such as for example Atheriosclerosis, Shoke, Hypertonie, coronary syndroms, cancer, thrombolylic diseases, affected wound healing and diseases accompanied by increased cell death. In another aspect of the invention, such pharmaceutical can be used for the prevention and/or treatment of diseases associated with a dysregulation of angiogenesis, such as for example tumor growth, rheumatical arthritis and diabetic setinopathy.

The study, reported about the cloning, chromosomal mapping, tissue expression and functional identification as a receptor for S1P of a novel GPCR, EDG8, the fifth functional receptor for sphingosine 1-phosphate.

In an effort to identify new G-protein coupled receptors of the EDG-family a database search with alignments of the currently known 18 members of this receptor family was performed, comprising human EDG1-7 sequences up to the putative EDGs from Xenopus and Zebra-fish. A multiple alignment of these sequences was created by CLUSTALW and used in a PSI-BLAST search to scan translated versions of human genomic DNA sequences, which were publicly available in the different EMBL sections. For translation of DNA into protein sequences, individual protein files within two respective STOP-codon were created and all proteins shorter than 50 amino acids were ignored. As the majority of GPCRs is unspliced searching for GPCRs within genomic sequences should bring about novel receptor proteins.
Performing a PSI-BLAST search, the various cDNAs and genomic contigs, respectively, for the human EDG1-7 receptors were identified, and an additional genomic hit, highly homologous to human EDG5 (51% homology), termed EDG8. The nucleotide and amino acid sequence of the new putative GPCR are depicted in Fig.1A.

Hydropathy analysis (hydrophobicity plot not shown) suggests a seven transmembrane protein with three alternating extra- and intracellular loops, assumed to be the heptahelix structure common to GPCRs.

To shed more light on the relationships involved in the molecular evolution of the EDG-receptor family, a grow tree phylogram was constructed using the neighbor joining method (GCG software) (Fig.1B) (Comparison of amino acid sequences). According to this phylogenetic tree, the human EDG-family can be divided into two distinct groups: EDG1, 3, 5 and 6 belonging to one, EDG2, 4 and 7 belonging to the other group. These two groups are discriminated further by their preference for different lipid ligands: EDG1, 3, 5, 6 are preferentially stimulated by sphingosin 1-phosphate (S1P) (Yatomi et al., 1997b; Lee et al., 1998a,b; Ancellin and Hla, 1999; Yamazaki et al., 2000; Van Brocklyn et al., 2000), EDG2, 4 and 7 by lysophosphatidic acid (LPA) (Hecht et al., 1996; An et al., 1998; Im et al., 2000 ). The newly identified EDG8 exhibited *highest similarity (86.8% aminoacid identity) to the rat nrg1-protein* (Fig. 1B), a GPCR recently cloned by EST-expression profiling from a rat PC12 cell library (Glickman et al., 1999), which probably represents the rat homologue of human EDG8. In the report of Glickman, however, the authors did not address the question of the activating ligand of this receptor. The high similarity between EDG8 and the known sphingosin 1-phosphate (S1P) receptors EDG1, 3 and 5 (48-51%) (Fig. 1C) led to test the hypothesis that EDG8 may be a functional S1P-receptor.

In testing for S1P receptor activity, the EDG8 cDNA was introduced into chinese hamster ovary (CHO) cells by transient transfection. CHO cells were chosen as they *exhibit minimal responses to sphingosin 1-phosphate in concentrations up to 1 μM* but respond to S1P after transfection with the S1P preferring receptors EDG 1, 3 and 5 (Okamoto et al., 1998; Kon et al., 1999). To test functional receptor activity the mobilization of $[Ca^{2+}]_i$ was monitored for three reasons:

1.) S1P has been reported to increase $Ca^{2+}$ in many cell types (Ghosh et al., 1990; Zang et al., 1991; Durieux et al., 1993; Chao et al., 1994; Gosh et al., 1994; Mattie et al., 1994; Meyer zu Heringdorf et al., 1996; Okajima et al., 1996; van Koppen et al., 1996; Törnquist et al., 1997; Yatomi et al., 1997; Noh et al., 1998; An et al., 1999 )

2.) the identification of EDG1, 3, 5 and 6 as receptors for S1P has provided the molecular basis for a GPCR mediated mechanism and the receptors are known to

mediate intracellular $Ca^{2+}$-release through either PTX-sensitive $G\alpha_i$ proteins or the PTX-insensitive $G\alpha_{q/11}$ pathway (Okamoto et al., 1998; Kon et al., 1999; Gonda et al., 1999)

3.) all currently known S1P-responding EDG-receptors (except EDG6) are present in endothelial cells (A. Niedernberg et al., submitted), in which intracellular $Ca^{2+}$ release is a major pathway in the generation of NO, an important factor in vascular biology. Thus, identification of the complete set of S1P receptors, involved in intracellular $Ca^{2+}$ mobilization could help clarify the role of the individual subtypes in endothelial cell signalling.

Fig.2 depicts measurement of the intracellular $Ca^{2+}$ concentration, mediated by S1P via the putative S1P receptor EDG8. For sake of comparison, the S1P-receptors EDG1, 3, 5, and 6, which have been reported to mobilize $[Ca^{2+}]_i$, were included. $[Ca^{2+}]_i$ were recorded as real time measurements using the Fluorescence plate imaging reader (FLIPR, Molecular Devices). Initially, CHO cells transfected with empty vector DNA were stimulated with different concentrations of S1P (10, 100, 1000 nM). None of the applied S1P concentrations was capable of eliciting significant rises in intracellular $Ca^{2+}$ (Fig. 2A), suggesting that S1P receptors are not expressed in CHO cells or, if expressed, are unable to signal via the endogeneous $G\alpha_q$ pathway. To address this issue, the G protein chimera $G\alpha_{qi5}$, which confers onto Gi coupled receptors the ability to stimulate the Gq pathway, and $G\alpha_{16}$, which links Gi- and Gs coupled receptors to PLCß and subsequent intracellular $Ca^{2+}$-mobilization were used. Upon stimulation with S1P, $G_{qi5}$- and $G_{16}$- transfected CHO cells did not give rise to significant increases in $[Ca^{2+}]_i$ (Fig. 2A). However, transient transfection of CHO-cells with the cDNAs coding for the EDG1, 3 and 5 receptor conferred S1P-responsiveness to the cells: it was confirmed that EDG1, 3 and 5 mobilize $[Ca^{2+}]_i$ in response to S1P (Fig. 2B, C, D) (Kon et al., 1999). As already known for a large number of Gq-coupled receptors, coexpression of $G\alpha_q$ augments the EDG1 and 5-mediated $Ca^{2+}$-response as compared with the $Ca^{2+}$ signal induced by stimulation of endogeneous $G\alpha_q$. In

case of EDG3, additional exogeneously added $G\alpha_q$ did not further improve the signal intensity. These results are in agreement with the findings reported by Kon et al. (1999), who showed that the EDG3-subtype causes the most robust enhancement of intracellular $Ca^{2+}$.

In case of EDG6, Yamazaki et al. (2000) obtained an S1P-induced mobilization of $[Ca^{2+}]_i$ but we failed to detect a significant $Ca^{2+}$ increase above basal levels in the absence of any cotransfected G-protein α subunit (Fig. 2E). The reason for this discrepancy could be the cellular background (CHO cells in this study vs. K562 cells in Yamazaki et al.), as they reported a pertussis toxin (PTX)-sensitive $Ca^{2+}$-response, indicating the involvement of Gi-type G-proteins. In this case the $Ca^{2+}$ signal would be elicited by ßγ, released from activated $G\alpha_i\beta\gamma$ heterotrimers. The $G\alpha_i$-induced $Ca^{2+}$ signals are known to be much smaller in intensity as compared with the $Ca^{2+}$ signals induced by bona-fide Gq-linked receptors (Kostenis et al., 1997). It may be that detection of such $[Ca^{2+}]_i$ concentrations is beyond the sensitivity of the FLIPR system.

EDG8 did not release $[Ca^{2+}]_i$ when stimulated with S1P (10, 100, and 1000 nM) (Fig.2F), but gained the ability to mobilize $Ca^{2+}$ upon cotransfection with $G\alpha_{16}$, a G-protein α subunit, known to couple GPCRs from different functional classes to the Gq-PLCß pathway or $G\alpha_{qi5}$, a mutant G-protein α subunit that confers onto Gi-linked receptors the ability to stimulate Gq (Conklin et al., 1993). These results show that EDG8 is a functional receptor for S1P and that EDG8-induced $Ca^{2+}$ responses are due to a non-Gq pathway, probably the activation of phospholipase Cß2 by ßγ subunits of the Gi proteins. Furthermore, these results provide additional evidence that the S1P-preferring EDG-receptors couple differentially to the Gq and Gi pathways: EDG3 ist the most potent $Ca^{2+}$-mobilizing receptor and overexpression of $G\alpha_q$ does not further improve $Ca^{2+}$ signalling; EDG1 and 5 induce moderate $Ca^{2+}$-increases, that can be significantly improved by cotransfection of $G\alpha_q$ or a chimeric $G\alpha_{qi5}$ protein; EDG8-mediated $Ca^{2+}$-responses require cotransfection of $G\alpha_{qi5}$ or $G\alpha_{16}$.

To check, whether the EDG8 receptor also reacts to related lysophospholipid mediators, we examined the abilities of lysophosphatidic acid (LPA), dihydrosphingosin

1-phosphate (DHS1P), sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC) to increase intracellular $Ca^{2+}$ in CHO cells transiently transfected with the EDG8 receptor and the G-protein α subunits $G\alpha_{16}$ and $G\alpha_{qi5}$ (Fig.3). Besides S1P, which was the most potent activator of EDG8, LPA and DHS1P evoked $[Ca^{2+}]_i$ increases in concentrations of 100 and 1000 nM. SPC and LPC, respectively, failed to generate any significant response in concentrations up to 1 μM. These data show that EDG8 is a S1P preferring receptor, but also responds to related phospholipids like DHS1P or LPA, as has also been reported for EDG1, which is a high affinity receptor for S1P and a low affinity receptor for LPA (Lee et al., 1998b). Therefore, EDG8 receptor has the characteristic functionality to respond to S1P and related phospholipids like DMS 1P or LPA. The response to S1P and other related phospholipides can for example be determined as described in Example 3. Cells containing the respective Gα can be obtained as described in Example 2.

Next, the expression pattern of the EDG8 gene in human tissues was investigated by Northern blot analysis (Fig.4). Tissues positive for EDG8 RNA were skeletal muscle, heart and kidney, lower abundance of RNA was seen in liver and placenta, no signal was detected in brain, thymus, spleen, lung and peripheral blood leukocytes. In all tissues a single RNA transcript of 5.5 kb was observed after hybridization with a DIG-labelled EDG8 antisense RNA probe. EDG8 exhibits highest similarity to the rat nrg1-GPCR (Glickman et al., 1999) with an amino acid identity of 86.8% (Fig.1B) suggesting that it may be the human homolog of the rat nrg1 protein. However, the expression pattern of human EDG8 is quite different from the rat nrg1-receptor,

which is found almost exclusively in brain (Glickman et al., 1999). This finding suggests that EDG8 may represent a closely related but entirely different receptor from nrg1, rather than the human homolog. Never the less, it does not rule out the possibility that EDG8 and nrg1 are homologs with entirely different, species-dependent expression patterns.

As the first member of the EDG-family of GPCRs - EDG1 - was originally cloned as an endothelial differentiation gene from phorbol-myristic-acetate-treated differentiating

human endothelial cells (Hla and Maciag, 1990) and subsequently cloned from a human umbilical vein endothelial cell library exposed to fluid shear stress as an upregulated gene it is reasonable to assume that EDG receptors play an important role in the regulation of endothelial function. Therefore, the presence of EDG8 transcripts in several human endothelial cell lines was analyzed. RT-PCR analysis of human umbilival vein endothelial cells (HUVECs), human coronary artery endothelial cells (HCAECs), human microvascular endothelial cells of the lung (HMVEC-L) and human pulmonary artery endothelial cells (HPAEC) revealed EDG8 expression in all cell lines tested (Fig.5A). In Fig.5B it is shown that EDG8 specific primers indeed solely amplify EDG8 sequences and none of the related EDG1-7 sequences. These findings suggest that the presence of EDG8 in different peripheral organs may be due to its localization in endothelial cells; it does not rule out, however, that EDG8 transcripts occur in cell types other than endothelial cells.
The expression of EDG8 in addition to EDG1, 3, and 5 (Rizza et al., 1999) in HUVECS and several other endothelial cell lines is intriguing in view of all the reports regarding S1P effects on endothelial cell signalling. Hisano et al. (1999) reported that S1P protects HUVECS from apoptosis induced by withdrawal of growth factors and stimulates HUVEC DNA synthesis; the authors derived a model for cell-cell interactions between endothelial cells and platelets but the S1P-receptor responsible for HUVEC-protection of apoptosis could not be identified. Rizza et al., 1999 reported that S1P plays a role in endothelial cell leukocyte interaction in that S1P induces expression of cell adhesion molecules in human aortic endothelial cells, allowing monocytes and neutrophils to attach. These effects were blocked by pertussis toxin, suggesting the involvement of a Gi-coupled S1P receptor. The responsible S1P-receptor subtype, however, could not be identified and the EDG8 receptor was not included at the time of this study. Expression profiling of all EDG receptors in individual cell lines and the use of EDG receptor subtype selective compounds will clearly be necessary to help determine the role of the individual S1P receptors in endothelial cell signalling mechanisms.

Finally, the mapping of EDG receptors in genomic sequences allowed to derive the chromosomal localization for four genes of this family (Tab.1). Interestingly, so far, four

EDG-receptors including EDG8 are located on chromosome 19. In addition, the genomic sequence allowed the determination of the structure of the genes: the S1P-preferring receptors EDG1, 3, 5 and 8 are intronless as opposed to the LPA-preferring subtypes 2, 4 and 7, that contain an intron in the open reading frame in TMVI. These data suggest that in addition to the activating ligand and the degree of homology, the two subclasses of lysophospholipid receptors can be discriminated further by their genomic structure. The genomic structure of new potential EDG/LPA-receptor family members may also help predict the nature of the activating lipid ligand.

In conclusion, a new member of the EDG-family of G-protein coupled receptor, human EDG8, was isolated. This receptor functions as a cellular receptor for sphingosine 1-phosphate. EDG8 could exclusively be detected in peripheral tissues like skeletal muscle, heart and kidney and several human endothelial cell lines. It is conceivable that the expression in endothelial cells may account for the broad tissue distribution of this receptor. The existence of at least eight EDG-receptors for lysophospholipids suggests that receptor subtype selective agonists and antagonists will essentially be necessary for a better understanding of the biology of lysophospholipids and their respective receptors.

Figure legends

Fig.1A: The nucleotide and deduced amino acid sequence of human EDG8. The deduced amino acid sequence is shown below the nucleotide sequence with the nucleotide positions indicated on the left.

Fig. 1B: Phylogenetic tree of the EDG-family of receptors. The phylogenetic tree depicted was derived by the neighbor joining method method performed with the GCG program.

Fig.1C: Alignment of the amino acid sequence of human EDG8 with the other EDG-family members. The amino acid sequence of EDG8 is compared with the EDG1-7 polypeptides (EDG1: accession number M 31210, EDG2: accession number U 80811,

EDG3: accession number X 83864, EDG4: accession number AF 011466, EDG5: accession number AF 034780, EDG6: AJ 000479, EDG7: accession number AF 127138). The approximate boundaries of the seven putative transmembrane domains are boxed. Gaps are introduced to optimize the alignment.

Fig.2A-F: Mobilization of intracellular $Ca^{2+}$ by S1P (10, 100 and 1000 nM) mediated by the EDG1, 3, 5, 6 and 8 receptor in CHO cells, cotransfected with empty vector DNA as a control or the indicated G-protein α subunits.
A: S1P-induced $Ca^{2+}$-response in CHO cells transfected with vector DNA alone or the G protein α subunits Gq, G16 and Gqi5. B-F: S1P-induced $Ca^{2+}$-response in CHO cells transfected with the indicated EDG-receptor subtypes. Agonist-mediated changes of intracellular $Ca^{2+}$ were measured with the FLIPR using the $Ca^{2+}$-sensitive dye FLUO4 as described in Experimental procedures. Fluorescence of transfected cells loaded with FLUO4 was recorded before and after addition of S1P, applied in the indicated concentrations. Data are expressed as means of quadruplicate determinations in a single experiment. An additional experiment gave similar results.

Fig.3: Effects of S1P, LPA and related lysophospholipid mediators on EDG8-mediated increase in intracellular $Ca^{2+}$. CHO-cells were cotransfected with EDG8 and the G protein α subunits Gqi5 (upper panel) and G16 (lower panel) and rises in $[Ca^{2+}]_i$ were recorded with the FLIPR as described in Experimental procedures. The different lipids were applied in concentrations of 10, 100 and 1000 nM, respectively. Data are means of quadruplicate determinations of a representative experiment. Two additional experiments gave similar results.

Fig.4: Northern blot analysis of EDG8 in human tissues. Poly(A)+ RNA (1μg) from various human tissues (human multiple tissue Northern blots, CLONTECH) was hybridized with probes specific to human EDG8 (upper panel) and ß-actin (lower panel) on a nylon membrane. The origin of each RNA is indicated at the top, the molecular mass of standard markers in kilobases (kb) is shown on the left.

Fig.5A: Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of EDG8 in different human endothelial cell lines (HUVECS: human umbilical vein endothelial cells; HCAEC: human coronary artery endothelial cells; HMVEC-L: human microvascular endothelial cells from lung; HPAEC: human pulmonary artery endothelial cells). EDG8-specific transcripts were detected in all endothelial cell lines. Agarose gel electrophoresis of the PCR products after 35 cycles of amplification with the GC-melt kit (as described in Experimental Procedures) is shown. Amplification with EDG8-specific primers yields a 522 bp EDG8-fragment as indicated by the arrow. The EDG8 plasmid served as a template for the positive control, $H_2O$ was used instead of plasmid DNA as a negative control.

Fig.5B: PCR analysis of EDG8 primers for specificity of amplification of EDG8 sequences. Primers, specific for the EDG8 sequence, were checked for potential amplification of the related EDG1-7 sequences, using the respective plasmids as templates. Agarose gel electrophoresis of the PCR products after 35 cycles of amplification with the GC-melt kit (as described in Experimental Procedures) is shown. The EDG8 specific 522 bp band occurred only when EDG8 was used as a template. $H_2O$ was used instead of plasmid DNA as a negative control.

Fig.6: Experiments were performed according to example 3. Instead of lipids, a lipid library was used.

Fig.6A+B: Library plattes with rat EDG8 (r EDG8) and qi5.

Fig.6A: qi5 background.

Fig.6B: Measurement with rEDG8.

Fig.6C: Fluorescence change counts.

Fig.7: Experiments were performed according to example 3. Instead of Lipids, a lipid library was used.

Fig.7A+B: Library plates with human EDG8 (hEDG8) and qi5.

Fig.7A: q15 background.

Fig.7B: Measurement with hEDG8

Fig.7C: Fluourescence change counts.

Fig.8: Antagonism of S1P activation of rat and human EDG8.

Transiently transfected CHO cells expressing rat EDG8 and $G\alpha_{qi5}$ (A) and HEK 293 cells expressing human EDG8 and $G\alpha_{qi5}$ (B) were incubated with test compounds, namely , 0.1 µM Leukotriene B4, 1 µM 2-DHLA-PAF (1-O-Hexadecyl-2-O-dihomo-γ-linolenoyl-sn-glycero-3-phophorylcholine), 1µM $C_2$ Dihydroceramide, 0.1µM 15(S) HEDE (15(S)-Hydroxyeicosa-11Z,13E-dienoic acid), 1µM PAF C16 (1-O-Hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine), 1µM 16,16 Dimethyl $PGE_2$ (16,16-Dimethyl-Prostaglandin $E_{2)}$ 12, 0.1 µM (R)-HETE (12(R)-Hydroxyeicosa-5Z,8Z,10E,14Z-tetraenioc acid), 1µM 8-epi-$PGF_{2\alpha}$ (8epi-Prostaglandin $F_{2\alpha}$) 0.1 µM Leukotoxin A ((±) 9,10-EODE) or with solvent buffer for 3 min and then challenged with 1 µM S1P (sphingosine 1-phosphate). Peak fluorescence counts of cells preincubated with solvent buffer and then stimulated with 1 µM S1P were set 100 %. Fluorescence change counts were recorded with the FLIPR as described in detail in Experimetal procedures. Data are means ± SE of 2-3 independent experiments.

Fig.9: Inhibition of S1P mediated intracellular calcium release by suramin and NF023 (8,8`-(carbonylbis(imino-3,1-phenylene))bis-(1,3,5-naphatlenetrisulfonic acid)) in cells transiently cotransfected with with human EDG8 and $G\alpha_{qi5}$ (A) and rat EDG8 and $G\alpha_{qi5}$ (B). Transfected cells were first treated with the indicated concentrations of the inhibitor or solvent buffer for 3 minutes (NF023 and suramin did not show any effect on $[Ca^{2+}]_i$ mobilization during the preincubation period). Cells were then stimulated with

1µM S1P and in $[Ca^{2+}]_i$ measured with the FLIPR as described in the method section. Peak fluorescence counts were normalized and background responses of $G\alpha_{qi5}$ - transfected cells were subtracted. S1P-mediated calcium release in the absence of inhibitor was set 100%. Data are means ± SE of 4-7 independent experiments.

TABLE 1: Chromosomal localization, gene structure and accession number of the respective EDG genomic clones

Mapping of EDG receptors in genomic sequences allowed to derive a chromosomal assignment for EDG1, 2, 4-8. The chromosomal localization of EDG3 was obtained from Yamagutchi et al. (1996). Genomic sequences also revealed EDG1, 3, 5, 6 and 8 to be unspliced as opposed to EDG2, 4 and 7, which contain an intron in their open reading frame (ORF).

| EDG | Chromosomal localisation | spliced/unspliced in ORF | according BAC accession number: |
|---|---|---|---|
| EDG1 | 1p21.1-21.3 | unspliced | AL161741 |
| EDG2 | 9q31.1-32/ /18p11.3 | spliced | AL157881/ /AP000882 |
| EDG3 | 9q22.1-q22.2 | unspliced | |
| EDG4 | 19p12 | spliced | NT_000939 |
| EDG5 | 19 | unspliced | AC011511 |
| EDG6 | 19p13.3 | unspliced | AC011547 |
| EDG7 | 1p22.3-31.2 | spliced | AL139822 |
| EDG8 | 19 | unspliced | AC011461 |

## Examples

Example 1: Molecular cloning of the human EDG8 receptor.

As the putative human EDG8 sequence is intronless, we cloned the receptor from human genomic DNA (CLONTECH, Palo Alto, CA, 94303-4230) via polymerase chain reaction (PCR). PCR conditions, established to amplify the EDG8 sequence were 94°C, 1 min followed by 35 cycles of 94°C, 30sec, 68C, 3 min, using GC-Melt Kit (CLONTECH, Palo Alto,CA). Primers designed to amplify the EDG8 sequence contained a HindIII site in the forward, and a EcoRI site in the reverse primer, respectively. The 1197 bp PCR product was cloned into the pCDNA3.1(+) mammalian expression vector (Invitrogen, Carlsbad, California) and sequenced in both directions.

Example 2: Cell culture and Transfection.

CHO-K1 cells were grown in basal ISCOVE medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator. For transfections, 2 x $10^5$ cells were seeded into 35-mm dishes. About 24 hr later cells were transiently transfected at 50-80% confluency with the indicated receptor and G-protein constructs (1µg of plasmid DNA each) using the Lipofectamine transfection reagent and the supplied protocol (GIBCO). 18-24 hr after transfection cells were seeded into 96well plates at a density of 50.000 cells per well and cultured for 18-24 additional hr until used in the functional FLIPR assays.

The cDNA for Gα16 was cloned from TF1 cells by RT-PCR and ligated into the pCDNA1.1 mammalian expression vector (Invitrogen). Murine wild type Gαq was cloned from cells by RT-PCR and inserted into the BamHI-NsiI-sites of pCDNA1.1. To create the C-terminally modified $G\alpha_{qi5}$ subunit, in which the last five aa of wt Gαq were replaced with the correspoding $G\alpha_i$ sequence, a 175-bp BglII-NsiI fragment was replaced, in a two piece ligation, with a synthetic DNA fragment, containing the desired codon changes. The correctness of all PCR-derived sequences was verified by sequencing in both directions.

Example 3: Fluorometric Imaging Plate Reader (FLIPR) Assay.

Twenty-four hours after transfection, cells were splitted into 96-well, black-wall microplates (Corning) at a density of 50,000 cells per well. 18-24 hr later, cells were loaded with 95µl of HBSS containing 20 mM Hepes, 2.5 mM probenecid, 4 µM fluorescent calcium indicator dye Fluo4 (Molecular Probes) and 1% fetal bovine serum for 1 h(37°C, 5% $CO_2$). Cells were washed three times with HBSS containing 20 mM Hepes and 2.5 mM probenecid in a cell washer. After the final wash, the solution was aspirated to a residual volume of 100 µl per 96 well. Lipid ligands were dissolved in DMSO as 2 mM stock solutions (treated with ultrasound when necessary) and diluted at least 1:100 into HBSS containing 20 mM HEPES, 2.5 mM probenecid and 0.4 mg/ml fatty acid free bovine serum albumine. Lipids were aliquoted as 2X solutions into a 96 well plate prior to the assay. The fluorometric imaging plate reader (FLIPR, Molecular Devices) was programmed to transfer 100 µl from each well of the ligand microplate to each well of the cellplate and to record fluorescence during 3 min in 1 second intervals during the first minute and 3 second intervals during the last two minutes. Total fluorescence counts from the 18-s to 37-s time points are used to determine agonist activity. The instrument software normalizes the fluorescent reading to give equivalent initial readings at time zero.

Example 4: Northern Blot analysis.

Human multiple tissue Northern blots were purchased from CLONTECH (Palo Alto, CA, 94303-4230, USA) antisense RNA probes were generated by subcloning nucleotides 279-1197 of the coding region into the Bam HI-Eco RI sites of the expression vector PSPT18 (Roche Diagnostics, Mannheim, Germany) and subsequent random priming with a DIG-RNA Labeling kit (Roche Diagnostics, Mannheim, Germany), using T7 RNA polymerase. Hybridization was carried out at 68°C for 16 h in hybridization buffer (Dig Easy Hyb Roche Diagnostics, Mannheim, Germany). Each blot was washed , blocked and detected as indicated in the standard protocol with the DIG Wash and Block Buffer set (Roche Diagnostics, Mannheim, Germany) and treated with 1 ml CSPD ready–to-use(Roche Diagnostics, Mannheim, Germany) for 15 min , 37°C and developed for 5 min on the Lumiimager (Roche). Finally, each blot was

stripped (50 % formamid,5% SDS, 50 mM Tris/HCl pH 7,5 ; 80° C, 2x 1 hour) and rehybridized with a GAPDH antisense RNA probe as an internal standard.

Example 5: RNA Extraction and RT-PCR.

RNA was prepared from different endothelial cell lines (HUVECS, HCAEC, HMVEC-L, HPAEC) using the TRIzol reagent (Hersteller, Lok.). Briefly, for each endothelial cell line, cells of a subconfluent 25 cm2 tissue culture flask were collected in 2,5ml TRIzol and total RNAs were extracted according to the supplied protocol. The purity of the RNA preparation was checked by veryfying the absence of genomic DNA. An aliquot of RNA, corresponding to ~5µg, was used for the cDNA generation using MMLV reverse transcriptase and the RT-PCR kit from STRATAGENE. RT-PCR was carried out in a volume of 50 µl, the RT-PCR conditions were set to 65°C for 5 min, 15min at RT, 1 hour at 37°C, 5 min at 90°C, chill on ice.

The cDNA templates for the PCR reactions (35 cycles of 94°C for 30 sec, 68°C for 3 min) were the reverse transcribed products of RNAs isolated from human endothelial cell lines (HUVECS,HCAEC, HMVEC-L, HPAEC). Typically, 1-5 µl of reverse transcribed cDNAs were used as templates for the PCR reactions.

Example 6: Sources of materials.

1-oleoyl-LPA, sphingosin 1-phosphate (S1P), dihydrosphingosin 1-phosphate (DHS1P), lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC) and fatty acid free BSA were from SIGMA (P.O.Box 14508, St. Louis, Missouri 63178). CHO-K1 cells were obtained from the American Type culture collection (ATCC, Manassas, Virginia), cell culture media and sera from GIBCO BRL (Gaithersburg, MD), the Ca fluorescent dye FLUO4 and pluronic acid from Molecular devices (Sunnyvale CA 94089-1136,USA) human northern blot membrane from CLONTECH (1020 East Meadow Circle, Palo Alto, California 94303-4230, USA.), commercially available cDNAs (heart, fetal heart, left atrium, left ventricle, kidney, brain, liver, lung, aorta) from Invitrogen, oligonucleotides from MWG-Biotech AG (Ebersberg, Germany), the RT-PCR kit from SIGMA, the GC-melt PCR kit from Clontech (Palo Alto, CA), the expression plasmid pcDNA3.1 for EDG8 and pCDNA1.1 for expression of G-protein α

subunits from Invitrogen (Carlsbad, CA 92008), competent DH5α from GIBCO and MC 1063 from Invitrogen.

References

An S, BleuT, Hallmark OG, and Goetzl EJ (1998) Characterization of a novel subtype of human G protein coupled receptor for lysophosphatidic acid. J Bio. Chem 273:7906-7910

An S, Bleu T, and Zheng Y (1999) Transduction of intracellular calcium signals through G protein-mediated activation of phospholipase C by recombinant sphingosine 1-phosphate receptors. Mol Pharmaco. 55:787-794

An S, Zheng Y, and Bleu T (2000) Sphingosine 1-phosphate induced cell proliferation, survival, and related signaling events mediated ba G protein coupled receptors edg3 and edg5. J Biol Chem 275:288-296

Ancellin N and Hla T (1999) Differential pharmacological properties and signal transduction of the sphingosine 1-phosphate receptors EDG-1, EDG-3, and EDG-5. JJ Biol Chem 274:18997-19002

Bandoh K, Aoki J, Hosono H, Kobayashi S, Kobayashi T, Murakami-Murofushi K, Tsujimoto M, Arai H, and Inoue K (2000) J Biol Chem 274:27776-27785

Bünemann M, Liliom K, Brandts BK, Pott L, Tseng JL, Desiderio DM, Sun G, Miller D, and Tigyi G (1996) A novel membrane receptor with high affinity for lysosphingomyelin and sphingosine 1-phosphate in atrial myocytes. EMBO J 15:5527-5534

Chao CP, Laulederkind SJ, and Ballou LR (1994) Sphingosine mediated phosphatidyl metabolism and calcium mobilization. J Biol Chem 269:5849-5856

Durieux ME, Carlisle SJ, Salafranca MN, and Lynch KR (1993) Responses to sphingosine 1-phosphate in X. laevis oocytes: similarities with lysophosphatidic acid signalling. Am J Physiol 264:C1360-C1364

Glickman M, Malek RL, Kwitek-Black AE, Jacob HJ, and Lee NH (1999) Molecular cloning, tissue-specific ecpression, and chromosomal localization of a novel nerve growth factor-regulated G-protein-coupled receptor, nrg-1. Molecular and Cellular Neuroscience 14:141-152

Gohla A, Harhammer R, and Schultz G (1998) The G protein G13 but not G12 mediates signalling from lysophosphatidic acid receptor via epidermal growth factor to Rho..
J Biol Chem 273:653-4659

Gohla A, Offermanns S, Wilkie TM, and Schultz G (1999) Differential involvement of G 12 and G 13 in receptor-mediated stress fiber formation. J Biol Chem 274:17901-17907

Gonda K, Okamoto H, Takuwa N, Yatomi Y, Okazaki H, Sakrai T, Kimura S, Sillard R, Harii K, and Takuwa Y (1999) The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. Biochem J 337:67-75

Gosh TK, Bian J, and Gill DL (1994) Sphingosine 1-phosphate generated in the endoplasmic reticulum membrane activates release of stored Calcium. J Biol Chem 269:22628-22635

Gueguen G, Gaige B, Grevy JM, Rogalle P, Bellan J, Wilson M, Klaebe A, Pont F, Simon MF, and Chap H (1999) Structure-activity analysis of the effects of lysophosphatidic acid on platelet aggregation. Biochemistry 38:8440-8450

Hisano N, Yatomi Y, Satoh K, Akimoto S, Mitsumata M, Fujino MA, and Ozaki Y (1999)

Induction and suppression of endothelial cell apoptosis by sphingolipids: a possible in vitro model for cell-cell interactions between platelets and endothelial cells. Blood 93:4293-4299

Hla T and Maciag T (1990) An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. J. Biol. Chem. 265: 9308-9313

Hla T, Lee M, Ancellin N, Liu CH, Thangada S, Thompson BD, and Kluk M (1999) Sphingosine-1-phosphate: extracellular mediator or intracellular second messenger? Biochem Pharm 58:201-207

Im DS, Heise CE, Harding MA, George SR, O'Dowd BF, Theodorescu D, and Lynch KR (2000) Molecular cloning and characterization of a lysophosphatidic acid receptor, edg7, expressed in prostate. Mol Pharmacol 57:753-759

Jalink K, Moolenaar WH, and van Dujin B (1993) Lysophosphatidic acid is a chemoattractant for dictyostelium discoideum amoebae. Proc Natl Acad Sci USA 90:1857-1861

Jalink K, Hordijk PL, and Moolenaar WH (1994) Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. Biochim Biophys Acta 1198:185-196

Kon J, Sato K, Watanabe T, Tomura H, Kuwabara A, Kimura T, Tamama K, Ishizuka T, Murata N, Kanda T, Kobayashi I, Ohta H, Ui M, and Okajima F (1999) Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signalling pathways in their cDNA-transfected chinese hamster ovary cells. J Biol Chem 274:23940-23947

Kostenis E, Degtyarev MY, Conklin BR, and Wess J (1997) The N-terminal extension of G q is critical for constraining the selectivity of receptor coupling. J Biol Chem 272:19107-19110

Lee MJ, Evans M, and Hla T (1996) The inducible G protein-coupled receptor edg-1 signals via the G(i)/mitogen-activated protein kinase pathway. J Biol Chem 271:11272-11279

Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev R, Spiegel S, and Hla T (1998a) Sphingosine 1-phosphate as a ligand for the G protein coupled receptor EDG-1. Science 279:1552-1555

Lee MJ, Thangada S, Liu CH, Thompson BD, and Hla T (1998) Lysophosphatidic acid stimulates the G-protein-coupled receptor edg-1 as a low affinity agonist. J Biol Chem 273:22105-22112

Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, and Hla T (1999) Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine 1-phosphate. Cell 99:301-312

Lynch K and Im DS Life on the edg. Trends Pharmacol Sci 20:473-475

Mattie M, Brooker G, and Spegel S (1994) Sphingosine 1-phosphate, a putative second messenger, mobilizes Calcium from internal stores via an inositoltriphosphate-independent pathway. J Biol Chem 269:3181-3188

Meyer zu Heringdorf D, van Koppen CJ, Windorfer B, Himmel HM, and Jakobs KH (1997) Calcium signalling by G protein coupled sphingolipid receptors in bovine aortic endothelial cells. Naunyn-Schmiedeberg's Arch Pharmacol 354:397-403

Moolenaar WH, Kranenburg O, Postma FR, and Zondag GCM (1997) Lysophosphatidic acid: G-protein signalling and cellular responses. Current opinion in cell biology 9:168-173

Morris AJ (1999) One wheel on my wagon: lysolipid phosphate signalling. Trends Pharmacol Sci 20:393-395

Noh SJ, Kim MJ, Shim S, and Han JK (1998) Different signalling pathway between sphingosine 1-phosphate and lysophosphatidic acid in Xenopus oocytes: Functional coupling of the sphingosine 1-phosphate receptor to PLCx-beta in Xenopus oocytes. J Cell Physiol 176:412-423

Okajima F, Tomura H, Sho K, Nochi H, Tamoto K, and Kondo Y (1996) Involvement of pertussis toxin-sensitive GTP-binding proteins in sphingosine 1-phosphate induced activation of phospholipase C-$Ca^{2+}$ system in HL60 leukemia cells.
FEBS Lett 379:260-264

Okamoto H, Takuwa N, Gonda K, Okazaki H, Chang K, Yatomi Y, Shigematsu H, and Takuwa Y (1998) EDG1 is a functional sphingosine 1-phosphate receptor that is linked via a Gi/o to multiple signalling pathways, including phospholipase C activation, $Ca^{2+}$-mobilization, ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. J Biol Chem 273:27104-27110

Postma R, Jalink K, Hengeveld T, and Moolenaar WH (1996) Sphingosine 1-phosphate rapidly induces Rho-dependent neurite retraction: Action through a specific cell surface receptor. EMBO J 15:2388-2392

Rizza C, Leitinger N, Yue J, Fischr DJ, Wang D, Shih PT, Lee H, tigyi G, and Berliner JA (1999) Laboratory Investigation 79:1227-1235

Sadahira Y, Ruan F, Hakomori S, and Igarashi Y (1992) Sphingosine 1-phosphate, a specific endogeneous signalling molecule controlling cell motility and tumor cell invasiveness. Proc Natl Acad Sci USA 89:9686-9690

Schulze C, Smales C, Rubin LL, and Staddon JM (1997) Lysophosphatidic acid increases tight junction permeability in cultured brain endothelial cells. J Neurochem 68:991-1000

Siess W, Zangl KJ, Essler M, Bauer M, Brandl R, CorrinthC, Bittman R, Tigyi G, and Aepfelbacher M (1999) Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc Natl Acad Sci USA 96:6931-6936

Tokumura A, Fukuzawa K, Yamada S, and Tsukatani H (1980) Stimulatory effect of lysophosphatidic acids on uterine smooth muscles of non-pregnant rats. Arch Int Pharmacodyn Ther 245:74-83

Tokumura A,Yotsumoto T, Masuda Y, and Tanaka S (1995) Vasopressor effect of lysophosphatidic acid on spontaneously hypertensive rats and wistar kyoto rats. Research Communications in Molecular Patology and Pharmacology 90:96-102

Törnquist K, Saarinen P, Vainio M, and Ahlstrom M (1997) Sphingosine 1-phosphate mobilizes sequestered Calcium, activates calcium entry, and stimulates desoxyribonucleic acid synthesis in thyroid FRTL-5 cells. Endocrinology 138:4049-4057

Van Brocklyn JR, Graler MH, Bernhardt G, Hobson JP, Lipp M, Spiegel S (2000) Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6. Blood 95(8):2624-2629

Van Koppen C, Meyer zu Heringdorf D, Laser KT, Zhang C, Jakobs KH, Bunemann M, and Pott L (1996) Activation of a high affinity Gi protein-coupled plasma membrane receptor by sphingosine 1-phosphate. J Biol Chem 271:2082-2087

Wu J, Spiegel S, and Sturgill TW (1995) Sphingosine 1-phosphate rapidly activates the mitogen activated protein kinase pathway by a G protein-dependent mechanism. J Biol Chem 270:11484-11488

Xia P, Wang L, Gamble JR, and Vadas MA (1999) Activation of sphingosine kinase by tumor necrosis factor- inhibits apoptosis in human endothelial cells. J Biol Chem 274:34499-34505

Yamazaki Y, Kon J, Sato K, Tomura H, Sato M, Yoneya T, Okazaki H, Okajima F, Ohta H(2000) Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca(2+) signaling. Biochem Biophys Res Commun 268(2):583-589

Yatomi Y, Yamamura S, Ruan F, and Igarashi Y (1997a) Sphingosine 1-phosphate induces platelet activation through an extracellular action and shares a platelet surface receptor with lysophosphatidic acid. J Biol Chem 272:5291-5297

Yatomi Y, Igarashi Y, Yang L, Hisano N,Qi R, Asazuma N, Satoh K, Ozaki Y, and Kume S (1997b) J Biochem (Tokyo) 12:969-973

Zhang H, Desai NN, Olivera A, Seki T, Brooker G, and Spiegel S (1991) Sphingosine 1-phosphate, a novel lipid, involved in cellular proliferation. J Cell Biol 114:155-167

List of non-standard abbreviations:

S1P, sphingosine 1-phosphate; LPA, lysophosphatidic acid; dHS1P, dihydro sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; LPC, lysophosphatidylcholine; GPCR, G-protein-coupled receptor; G-protein, guanine nucleotide-binding protein; $[Ca^{2+}]_i$, intracellular Calcium concentration, RT-PCR, reverse transcription polymerase chain reaction; bp, base pair; ORF, open reading frame; EST, expressed sequence tag; FAF-BSA, fatty acid free bovine serum albumine; HUVECS. Human umbilical vein endothelial cells; HCAEC, human coronary artery endothelial cells; HMVEC-L, human microvascular endothelial cells from lung; HPAEC, human pulmonary artery endothelial cells.

Table 2:

SEQ ID NO. 1: Nucleotide sequence of human EDG8

1 ATGGAGTCGGGGCTGCTGCGGCCGGCGCCGGTGAGCGAGGTCATCGTCCTGCATTACAAC

61 TACACCGGCAAGCTCCGCGGTGCGCGCTACCAGCCGGGTGCCGGCCTGCGCGCCGACGCC

121 GTGGTGTGCCTGGCGGTGTGCGCCTTCATCGTGCTAGAGAATCTAGCCGTGTTGTTGGTG

181 CTCGGACGCCACCCGCGCTTCCACGCTCCCATGTTCCTGCTCCTGGGCAGCCTCACGTTG

241 TCGGATCTGCTGGCAGGCGCCGCCTACGCCGCCAACATCCTACTGTCGGGGCCGCTCACG

301 CTGAAACTGTCCCCCGCGCTCTGGTTCGCACGGGAGGGAGGCGTCTTCGTGGCACTCACT

361 GCGTCCGTGCTGAGCCTCCTGGCCATCGCGCTGGAGCGCAGCCTCACCATGGCGCGCAGG

421 GGGCCCGCGCCCGTCTCCAGTCGGGGGCGCACGCTGGCGATGGCAGCCGCGGCCTGGGGC

481 GTGTCGCTGCTCCTCGGGCTCCTGCCAGCGCTGGGCTGGAATTGCCTGGGTCGCCTGGAC

541 GCTTGCTCCACTGTCTTGCCGCTCTACGCCAAGGCCTACGTGCTCTTCTGCGTGCTCGCC

601 TTCGTGGGCATCCTGGCCGCTATCTGTGCACTCTACGCGCGCATCTACTGCCAGGTACGC

661 GCCAACGCGCGGCGCCTGCCGGCACGGCCCGGGACTGCGGGGACCACCTCGACCCGGGCG

721 CGTCGCAAGCCGCGCTCGCTGGCCTTGCTGCGCACGCTCAGCGTGGTGCTCCTGGCCTTT

781 GTGGCATGTTGGGGCCCCCTCTTCCTGCTGCTGTTGCTCGACGTGGCGTGCCCGGCGCGC

841 ACCTGTCCTGTACTCCTGCAGGCCGATCCCTTCCTGGGACTGGCCATGGCCAACTCACTT

901 CTGAACCCCATCATCTACACGCTCACCAACCGCGACCTGCGCCACGCGCTCCTGCGCCTG

961 GTCTGCTGCGGACGCCACTCCTGCGGCAGAGACCCGAGTGGCTCCCAGCAGTCGGCGAGC

1021 GCGGCTGAGGCTTCCGGGGGCCTGCGCCGCTGCCTGCCCCCGGGCCTTGATGGGAGCTTC

1081 AGCGGCTCGGAGCGCTCATCGCCCCAGCGCGACGGGCTGGACACCAGCGGCTCCACAGGC

1141 AGCCCCGGTGCACCCACAGCCGCCCGGACTCTGGTATCAGAACCGGCTGCAGACTGA

Table 3:

SEQ ID NO. 2: *Amino acid sequence of human EDG8*

M E S G L L R P A P V S E V I V L H Y N

Y T G K L R G A R Y Q P G A G L R A D A

V V C L A V C A F I V L E N L A V L L V

L G R H P R F H A P M F L L L G S L T L

S D L L A G A A Y A A N I L L S G P L T

L K L S P A L W F A R E G G V F V A L T

A S V L S L L A I A L E R S L T M A R R

G P A P V S S R G R T L A M A A A A W G

V S L L L G L L P A L G W N C L G R L D

A C S T V L P L Y A K A Y V L F C V L A

F V G I L A A I C A L Y A R I Y C Q V R

A N A R R L P A R P G T A G T T S T R A

R R K P R S L A L L R T L S V V L L A F

V A C W G P L F L L L L L D V A C P A R

T C P V L L Q A D P F L G L A M A N S L

L N P I I Y T L T N R D L R H A L L R L

V C C G R H S C G R D P S G S Q Q S A S

A A E A S G G L R R C L P P G L D G S F

S G S E R S S P Q R D G L D T S G S T G

S P G A P T A A R T L V S E P A A D *

Claims:

1. An isolated polynucleotide comprising a nucleotide sequence that has at least 90 % identity to a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or the corresponding fragment thereof; or a nucleotide sequence complementary to said nucleotide sequence.

2. The polynucleotide of claim 1 which is DNA or RNA.

3. The polynucleotide of claim 1 or 2, wherein said nucleotide sequence is at least 90 % *identical to that contained in* SEQ ID NO. 1.

4. The polynucleotide of claim 3 wherein said nucleotide sequence is contained in SEQ ID NO. 1.

5. The polynucleotide with sequence SEQ ID NO. 1.

6. The polynucleotide as claimed in claims 1 to 5, wherein said encoding nucleotide sequence encodes the polypeptide of SEQ ID NO. 2 or a fragment thereof.

7. The polynucleotide as claimed in claims 1 to 6 having almost the same biological functionality as EDG8.

8. EDG8 DNA or RNA molecule comprising an expression system wherein said expression system is capable of producing a polypeptide or a fragment thereof having at least 90 % identity with a nucleotide sequence encoding the polypeptide of SEQ ID NO. 2 or said fragment when said expression system is present in a compatible host cell.

9. A host cell comprising the expression system of claim 8.

10. A process for producing an EDG8 polypeptide or fragment comprising culturing a host cell as claimed in claim 9 under conditions sufficient for the production of said polypeptide or fragment.

11. The process of claim 10 wherein said polypeptide or fragment is expressed at the surface of said cell.

12. Cells produced by the process of claim 11.

13. The process of claim 10 which further includes recovering the polypeptide or fragment from the culture.

14. A process for producing a cell which produces a EDG8 polypeptide or a fragment thereof comprising transforming or transfecting a host cell with the expression system as claimed in claim 8 such that the host cell, under appropriate culture conditions, produces a EDG8 polypeptide or fragment.

15. EDG8 polypeptide or a fragment thereof comprising an amino acid sequence which is at least 90 % identical to the amino acid sequence contained in SEQ ID NO. 2.

16. Polypeptide of claim 15 which comprises the amino acid sequence of SEQ ID NO. 2, or a fragment thereof.

17. EDG8 Polypeptide or fragment prepared by the method of claim 13.

18. A process for diagnosing a disease or a susceptibility to a disease related to expression or acitivity of EDG8 polypeptide comprising:
    a) determining the presence or absence of mutation in the nucleotide sequence encoding said EDG8 polypeptide in the genome of said subject; and/or
    b) analyzing for the presence or amount of the EDG8 polypeptide expression in a sample derived from said subject.

19. A method for identifying compounds which bind to EDG8 polypeptide comprising:
    a) tacting a cell as claimed in claim 12 or a part thereof with a candidate compound; and
    b) assessing the ability of said candidate compound to bind to said cells.

20. The method as claimed in claim 19 which further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an agonist.

21. The method as claimed in claim 19 which further includes determining whether the candidate compound effects a signal generated by activation of the EDG8 polypeptide at the surface of the cell, wherein a candidate compound which effects production of said signal is identified as an antagonist.

22. An agonist identified by the method of claim 20.

23. An antagonist identified by the method of claim 21.

24. The method of claim 19 which further includes contacting said cell with a known agonist for said EDG8 polypeptide; and determining whether the signal generated by said agonist is diminished in the presence of said candidate compound, wherein a candidate compound which effects a diminution in said signal is identified as an antagonist for said EDG8 polypeptide.

25. A method as claimed in claim 24, wherein the known agonist is S1P, LPA and/or DHS1P.

26. An antagonist identified by the method of claim 24 or 25.

27. Method of preparing a pharmaceutical composition comprising

a) identifying a compound which is an agonist or an antagonist of EDG8,

b) preparing the compound, and

c) optionally mixing the compound with suitable additives.

28. Pharmaceutical composition prepared by a process of claim 27.

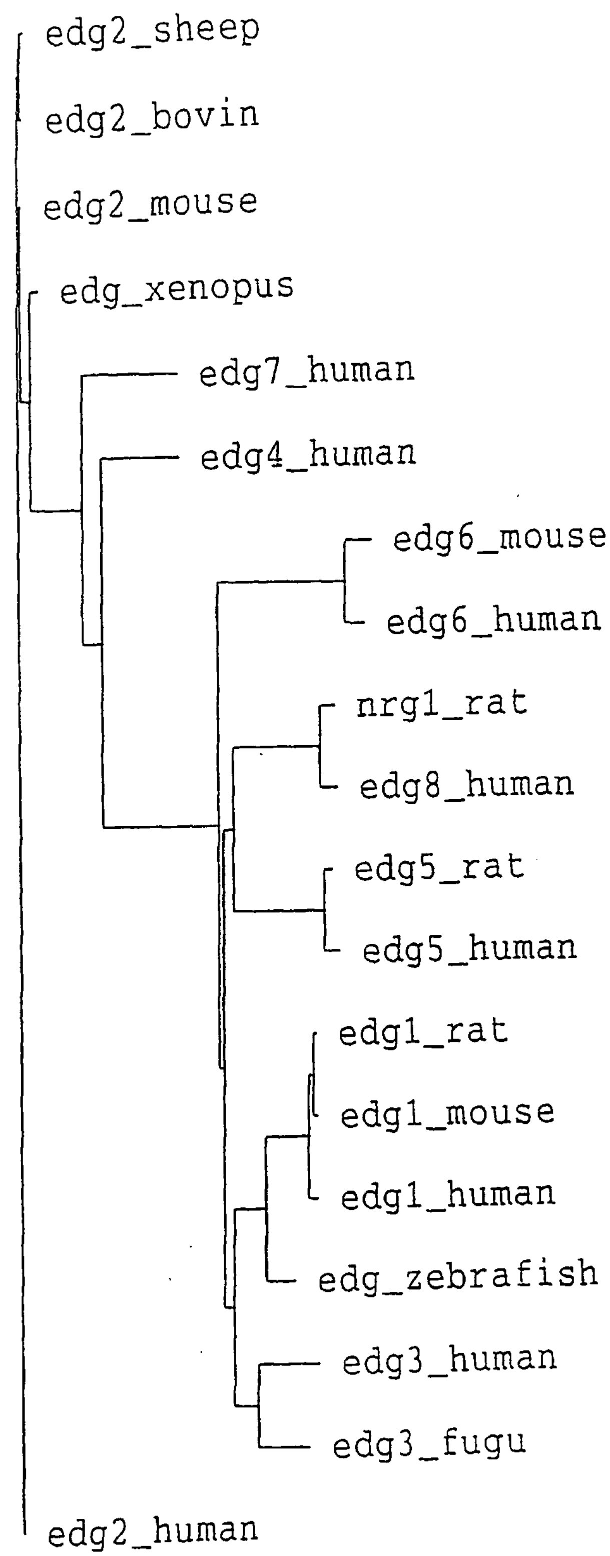
29. Pharmaceutical composition containing an EDG8 polypeptide or a part thereof having EDG8 functionality.

30. Pharmaceutical composition containing a polynucleotide encoding for EDG8 or a part thereof encoding for a peptide with EDG8 functionality.

FIG 1A:

```
   1 ATGGAGTCGGGGCTGCTGCGGCCGGCGCCGGTGAGCGAGGTCATCGTCCTGCATTACAAC
      M  E  S  G  L  L  R  P  A  P  V  S  E  V  I  V  L  H  Y  N

  61 TACACCGGCAAGCTCCGCGGTGCGCGCTACCAGCCGGGTGCCGGCCTGCGCGCCGACGCC
      Y  T  G  K  L  R  G  A  R  Y  Q  P  G  A  G  L  R  A  D  A

 121 GTGGTGTGCCTGGCGGTGTGCGCCTTCATCGTGCTAGAGAATCTAGCCGTGTTGTTGGTG
      V  V  C  L  A  V  C  A  F  I  V  L  E  N  L  A  V  L  L  V

 181 CTCGGACGCCACCCGCGCTTCCACGCTCCCATGTTCCTGCTCCTGGGCAGCCTCACGTTG
      L  G  R  H  P  R  F  H  A  P  M  F  L  L  L  G  S  L  T  L

 241 TCGGATCTGCTGGCAGGCGCCGCCTACGCCGCCAACATCCTACTGTCGGGGCCGCTCACG
      S  D  L  L  A  G  A  A  Y  A  A  N  I  L  L  S  G  P  L  T

 301 CTGAAACTGTCCCCCGCGCTCTGGTTCGCACGGGAGGGAGGCGTCTTCGTGGCACTCACT
      L  K  L  S  P  A  L  W  F  A  R  E  G  G  V  F  V  A  L  T

 361 GCGTCCGTGCTGAGCCTCCTGGCCATCGCGCTGGAGCGCAGCCTCACCATGGCGCGCAGG
      A  S  V  L  S  L  L  A  I  A  L  E  R  S  L  T  M  A  R  R

 421 GGGCCCGCGCCCGTCTCCAGTCGGGGGCGCACGCTGGCGATGGCAGCCGCGGCCTGGGGC
      G  P  A  P  V  S  S  R  G  R  T  L  A  M  A  A  A  A  W  G

 481 GTGTCGCTGCTCCTCGGGCTCCTGCCAGCGCTGGGCTGGAATTGCCTGGGTCGCCTGGAC
      V  S  L  L  L  G  L  L  P  A  L  G  W  N  C  L  G  R  L  D

 541 GCTTGCTCCACTGTCTTGCCGCTCTACGCCAAGGCCTACGTGCTCTTCTGCGTGCTCGCC
      A  C  S  T  V  L  P  L  Y  A  K  A  Y  V  L  F  C  V  L  A

 601 TTCGTGGGCATCCTGGCCGCTATCTGTGCACTCTACGCGCGCATCTACTGCCAGGTACGC
      F  V  G  I  L  A  A  I  C  A  L  Y  A  R  I  Y  C  Q  V  R

 661 GCCAACGCGCGGCGCCTGCCGGCACGGCCCGGGACTGCGGGGACCACCTCGACCCGGGCG
      A  N  A  R  R  L  P  A  R  P  G  T  A  G  T  T  S  T  R  A

 721 CGTCGCAAGCCGCGCTCGCTGGCCTTGCTGCGCACGCTCAGCGTGGTGCTCCTGGCCTTT
      R  R  K  P  R  S  L  A  L  L  R  T  L  S  V  V  L  L  A  F

 781 GTGGCATGTTGGGGCCCCCTCTTCCTGCTGCTGTTGCTCGACGTGGCGTGCCCGGCGCGC
      V  A  C  W  G  P  L  F  L  L  L  L  L  D  V  A  C  P  A  R

 841 ACCTGTCCTGTACTCCTGCAGGCCGATCCCTTCCTGGGACTGGCCATGGCCAACTCACTT
      T  C  P  V  L  L  Q  A  D  P  F  L  G  L  A  M  A  N  S  L

 901 CTGAACCCCATCATCTACACGCTCACCAACCGCGACCTGCGCCACGCGCTCCTGCGCCTG
      L  N  P  I  I  Y  T  L  T  N  R  D  L  R  H  A  L  L  R  L

 961 GTCTGCTGCGGACGCCACTCCTGCGGCAGAGACCCGAGTGGCTCCCAGCAGTCGGCGAGC
      V  C  C  G  R  H  S  C  G  R  D  P  S  G  S  Q  Q  S  A  S

1021 GCGGCTGAGGCTTCCGGGGGCCTGCGCCGCTGCCTGCCCCCGGGCCTTGATGGGAGCTTC
      A  A  E  A  S  G  G  L  R  R  C  L  P  P  G  L  D  G  S  F

1081 AGCGGCTCGGAGCGCTCATCGCCCCAGCGCGACGGGCTGGACACCAGCGGCTCCACAGGC
      S  G  S  E  R  S  S  P  Q  R  D  G  L  D  T  S  G  S  T  G
1141 AGCCCCGGTGCACCCACAGCCGCCCGGACTCTGGTATCAGAACCGGCTGCAGACTGA
      S  P  G  A  P  T  A  A  R  T  L  V  S  E  P  A  A  D  *
```

FIG 1B


edg2_sheep
edg2_bovin
edg2_mouse
edg_xenopus
edg7_human
edg4_human
edg6_mouse
edg6_human
nrg1_rat
edg8_human
edg5_rat
edg5_human
edg1_rat
edg1_mouse
edg1_human
edg_zebrafish
edg3_human
edg3_fugu
edg2_human

```
                1                                                          60
edg2_human   MAAISTSIPV ISQPQFTAMN EPQCFYNESI AFFYNRSGKH LAT.EWNTVS KLVMGL..GI
edg7_human   ---------- --------MN VE..CHYDKHM DFFYNRSNTD TVD.DW.TGT KLVIVLCVGT
edg4_human   ---------- -------MVI MGQCYYNETI GFFYNNSGKE LSS.HWR..P KDVVVVALGL
edg1_human   -----MGPTS VPLVKAHRSS VSDYVNYDII VRHYNYTGKL ..NISADKEN SIKLTSVVFI
edg3_human   ---------- --MATALPPR LQPVRGNETL REHYQYVGKL AGRLKEASEG S.TLTTVLFL
edg5_human   ---------- ------MGSL YSEYLNPNKV QEHYNYTKE. ..TLETQETT SRQVASAFIV
edg8_human   ---------- -----MESGL LRPAPVSEVI VLHYNYTGKL RG.ARYQPGA GLRADAVVCL
edg6_human   -----MNATG TPVAPESCQQ LAAGGHSRLI VLHYNHSGRL AGR.GGPEDG GLGALRGLSV

                61                                                        120
edg2_human   TVCIFIMLAN LLVMVAIYVN RRFHFPIYYL MANLAAADFF AGLAYFYLMF NTGPNTRRLT
edg7_human   FFCLFIFFSN SLVIAAVIKN RKFHFPFYYL LANLAAADFF AGIAYVFLMF NTGPVSKTLT
edg4_human   TVSVLVLLTN LLVIAAIASN RRFHQPIYYL LGNLAAADLF AGVAYLFLMF HTGPRTARLS
edg1_human   LICCFIILEN IFVLLTIWKT KKFHRPMYYF IGNLALSDLL AGVAYTANLL LSGATTYKLT
edg3_human   VICSFIVLEN LMVLIAIWKN NKFHNRMYFF IGNLALCDLL AGIAYKVNIL MSGKKTFSLS
edg5_human   ILCCAIVVEN LLVLIAVARN SKFHSAMYLF LGNLAASDLL AGVAFVANTL LSGSVTLRLT
edg8_human   AVCAFIVLEN LAVLLVLGRH PRFHAPMFLL LGSLTLSDLL AGAAYAANIL LSGPLTLKLS
edg6_human   AASCLVVLEN LLVLAAITSR MRSRRWVYYC LVNITLSDLL TGAAYLANVL LSGARTFRLA

                121                                                       180
edg2_human   VSTWLLRQGL IDTSLTASVA NLLAIAIERH ITVFR.MQLH TRMSNRRVVV VIVVIWTMAI
edg7_human   VNRWFLRQGL LDSSLTASLT NLLVIAVERH MSIMR.MRVH SNLTKKRVTL LILLVWAIAI
edg4_human   LEGWFLRQGL LDTSLTASVA TLLAIAVERH RSVMA.VQLH SRLPRGRVVM LIVGVWVAAL
edg1_human   PAQWFLREGS MFVALSASVF SLLAIAIERY ITMLK.MKLH NGSNNFRLFL LISACWVISL
edg3_human   PTVWFLREGS MFVALGASTC SLLAIAIERH LTMIK.MRPY DANKRHRVFL LIGMCWLIAF
edg5_human   PVQWFAREGS ASITLSASVF SLLAIAIERH VAIAK.VKLY GSDKSCRMLL LIGASWLISL
edg8_human   PALWFAREGG VFVALTASVL SLLAIALERS LTMAR.RGPA PVSSRGRTLA MAAAAWGVSL
edg6_human   PAQWFLREGL LFTALAASTF SLLFTAGERF ATMVRPVAES GATKTSRVYG FIGLCWLLAA

                181                                                       240
edg2_human   VMGAIPSVGW NCICDIENCS NMAPLYSDSY LVFWAIFNLV TFVVMVVLYA HIFGYVRQRT
edg7_human   FMGAVPTLGW NCLCNISACS SLAPIYSRSY LVFWTVSNLM AFLIMVVVYL RIYVYVKRKT
edg4_human   GLGLLPAHSW HCLCALDRCS RMAPLLSRSY LAVWALSSLL VFLLMVAVYT RIFFYVRRRV
edg1_human   ILGGLPIMGW NCISALSSCS TVLPLYHKHY ILFCTTVFTL LLLSIVILYC RIYSLVRTRS
edg3_human   TLGALPILGW NCLHNLPDCS TILPLYSKKY IAFCISIFTA ILVTIVILYA RIYFLVKSSS
edg5_human   VLGGLPILGW NCLGHLEACS TVLPLYAKHY VLCVVTIFSI ILLAIVALYV RIYCVVRSSH
edg8_human   LLGLLPALGW NCLGRLDACS TVLPLYAKAY VLFCVLAFVG ILAAICALYA RIYCQVRANA
edg6_human   LLGMLPLLGW NCLCAFDRCS SLLPLYSKRY ILFCLVIFAG VLATIMGLYG AIFRLVQASG

                241                                                       300
edg2_human   MRMSRHSSGP R......RNR DTMMSLLKTV VIVLGAFIIC WTPGLVLLLL D.VCCP..QC
edg7_human   NVLSPHTSGS I......SRR RTPMKLMKTV MTVLGAFVVC WTPGLVVLLL DGLNCR..QC
edg4_human   QRMAEHVSCH P......RYR ETTLSLVKTV VIILGAFVVC WTPGQVVLLL DGLGCE..SC
edg1_human   RRLTFR.... .KNISKASRS SENVALLKTV IIVLSVFIAC WAPLFILLLL DV.GCKVKTC
edg3_human   RKVANH.... .NN......S ERSMALLRTV VIVVSVFIAC WSPLFILFLI DV.ACRVQAC
edg5_human   ADMA...... .........A PQTLALLKTV TIVLGVFIVC WLPAFSILLL DY.ACPVHSC
edg8_human   RRLPARPGTA GTTSTRARRK PRSLALLRTL SVVLLAFVAC WGPLFLLLLL DV.ACPARTC
edg6_human   QKAP...... ...RPAARRK ARR..LLKTV LMILLAFLVC WGPLFGLLLA DVFGSNLWAQ

                301                                                       360
edg2_human   DVLAYEKFFL LLAEFNSAMN PIIYSYRDKE MSATFRQILC CQRSENPTGP TESSDRSASS
edg7_human   GVQHVKRWFL LLALLNSVVN PIIYSYKDED MYGTMKKMIC CFSQENP... ....ERRPSR
edg4_human   NVLAVEKYFL LLAEANSLVN AAVYSCRDAE MRRTFRRLLC CACLRQSTRE SVHYTSSAQG
edg1_human   DILFRAEYFL VLAVLNSGTN PIIYTLTNKE MRRAFIRIMS CCKCPSGD.. .........S
edg3_human   PILFKAQWFI VLAVLNSAMN PVIYTLASKE MRRAFFRLV. .CNC.LVR.. .........G
edg5_human   PILYKAHYFF AVSTLNSLLN PVIYTWRSRD LRREVLRPLQ CWRPGVGV.. .........Q
edg8_human   PVLLQADPFL GLAMANSLLN PIIYTLTNRD LRHALLRLVC CGRHSCGRDP SGS..QQSAS
edg6_human   EYLRGMDWIL ALAVLNSAVN PIIYSFRSRE VCRAVLSFLC CGCLRLGMRG PGDCLARAVE

                361                                                    418
edg2_human   LNHTILAGVH SNDHSVV--- ---------- ---------- ---------- --------
edg7_human   IPSTVLSRSD TGSQYIEDSI SQGAVCNKST S--------- ---------- --------
edg4_human   GASTRIMLPE NGHPLMTPPF SYLELQRYAA SNKSTAPDDL WVLLAQPNQQ D-------
edg1_human   AGKFKRPIIA GMEFSRSK.. .SDNSSHPQK DEGDNPETIM SSGNVNSSS- --------
edg3_human   RGARASPIQP ALDPSRSKSS SSNNSSHSPK VKEDLPHTDP SSCIMDKNAA LQNGIFCN
edg5_human   GRRRVGTPGH HLLPLRSSSS LERGMHMPTS PTFLEGNTVV ---------- --------
edg8_human   AAEASGGLRR CLPPGLDGSF SGSERSSPQR DGLDTSGSTG SPGAPTAART LVSEPAAD
edg6_human   AHSGASTTDS SLRP.RDSFR GSRSLSFRMR EPLSSISSVR SI-------- --------
```

Fig. 1C

FIG 2A

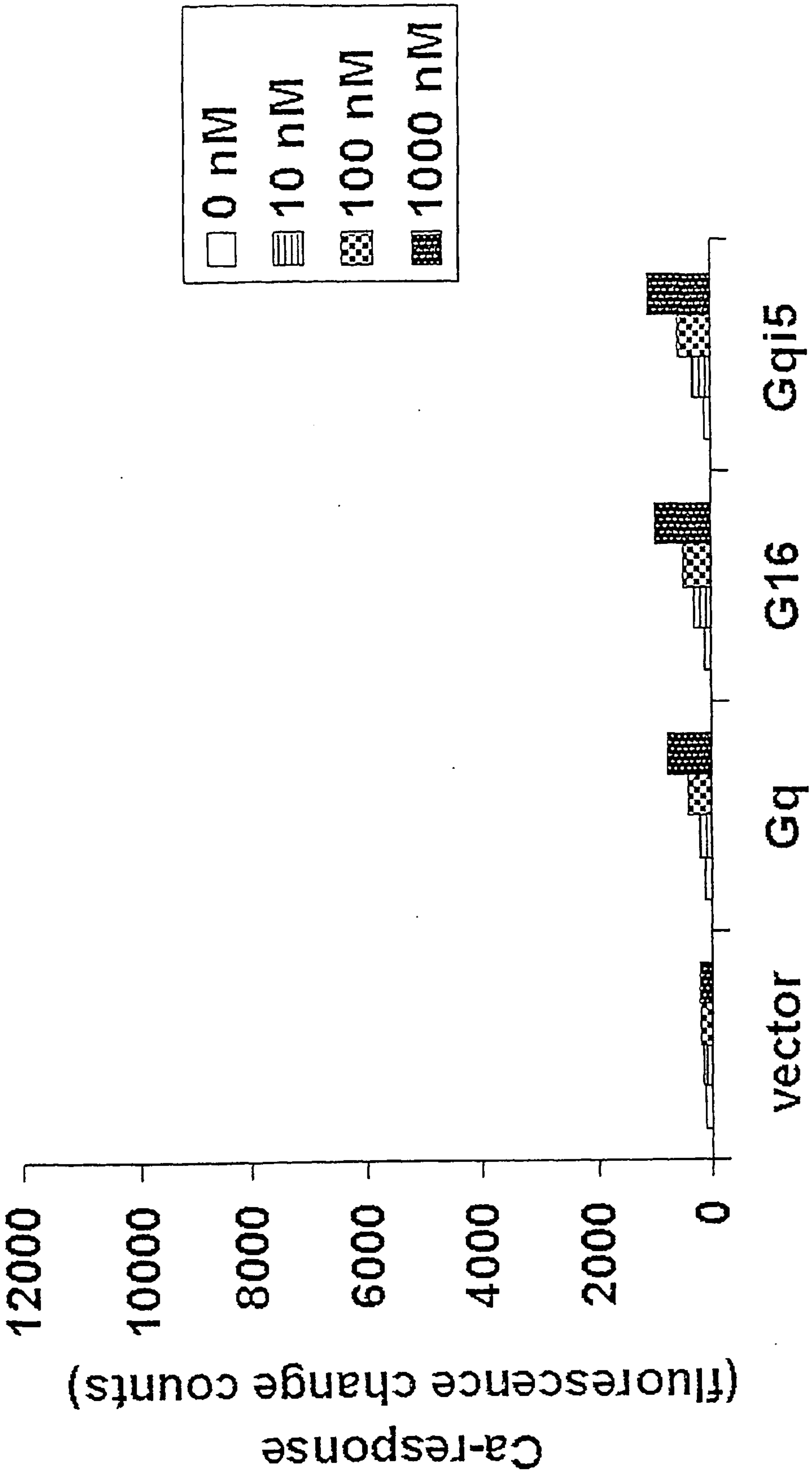

0 nM
10 nM
100 nM
1000 nM
vector
Gq
G16
Gqi5
0
2000
4000
6000
8000
10000
12000
Ca-response
(fluorescence change counts)

FIG 2B

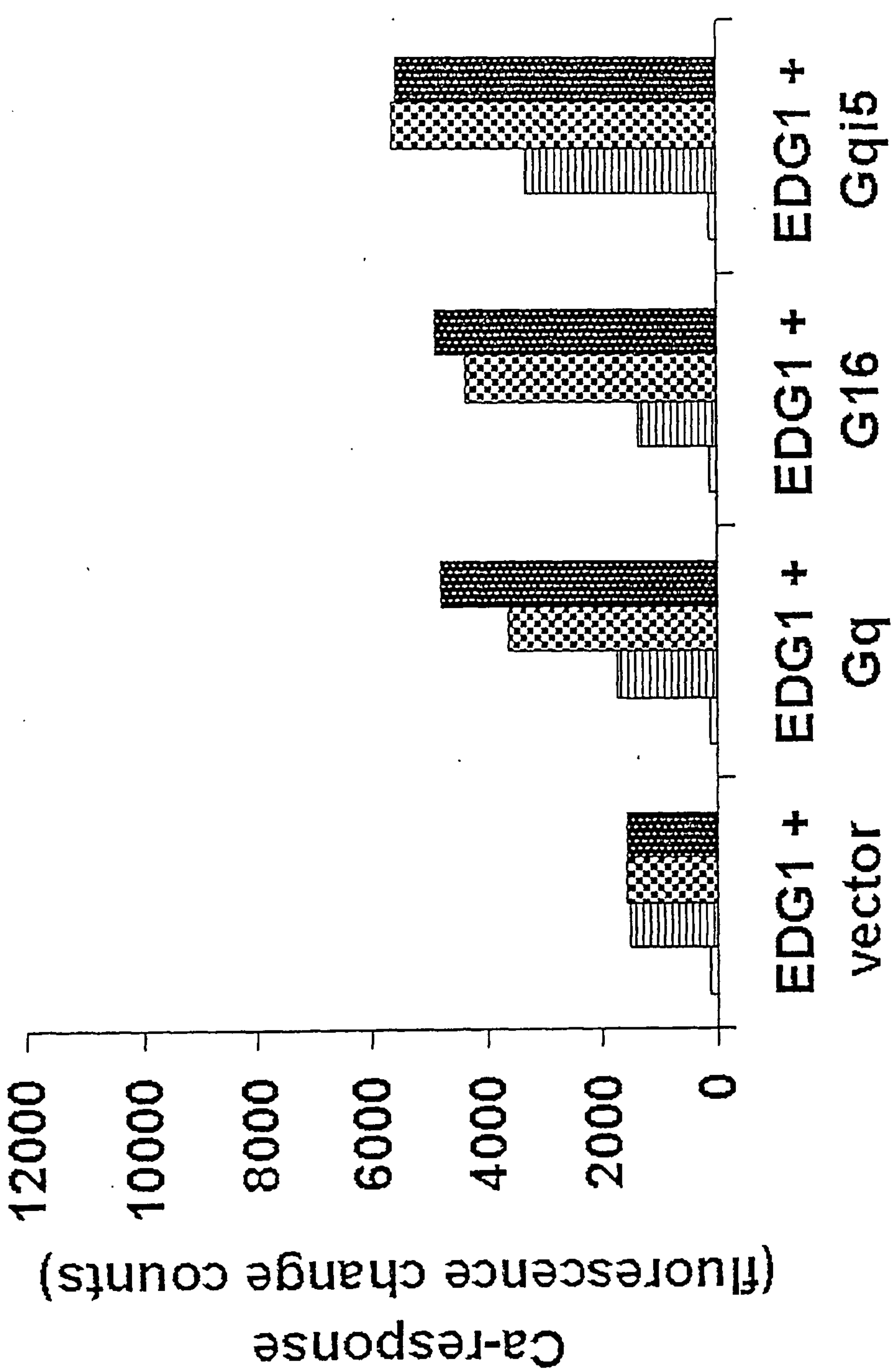

Ca-response
(fluorescence change counts)
0
2000
4000
6000
8000
10000
12000
EDG1 + vector
EDG1 + Gq
EDG1 + G16
EDG1 + Gqi5

FIG 2C

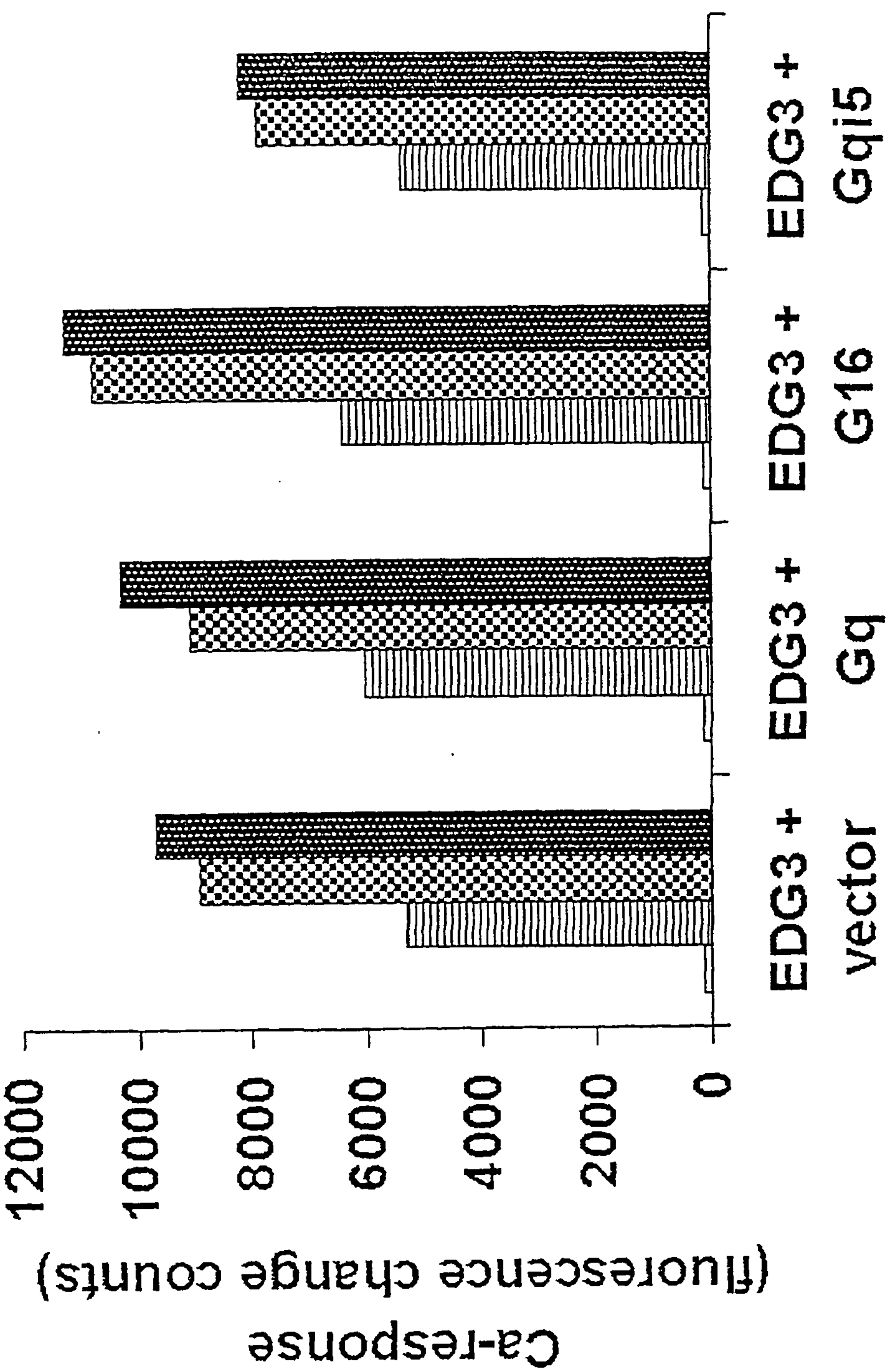

Ca-response
(fluorescence change counts)
0
2000
4000
6000
8000
10000
12000
EDG3 + vector
EDG3 + Gq
EDG3 + G16
EDG3 + Gqi5

FIG 2D

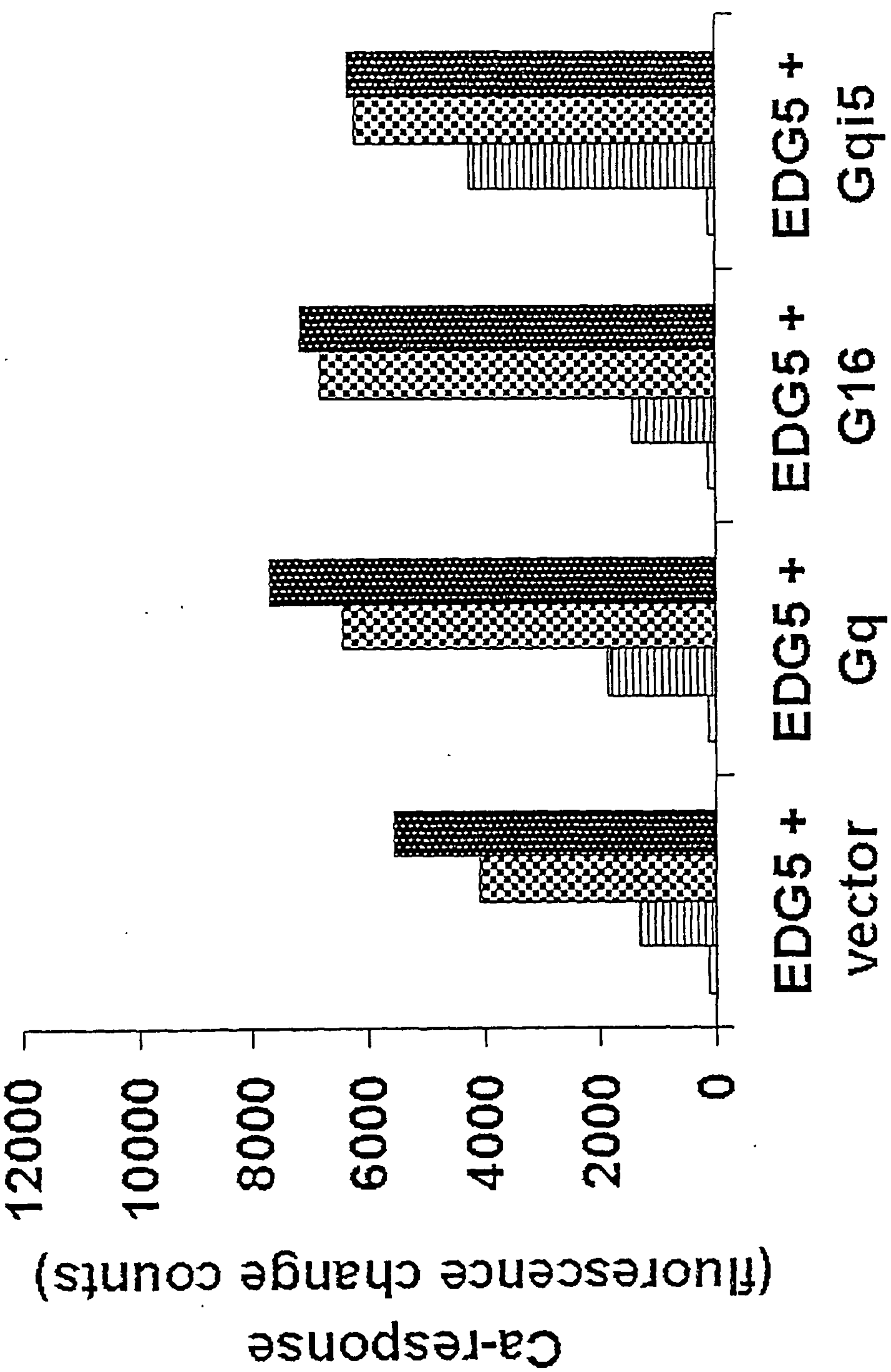

Ca-response
(fluorescence change counts)
12000
10000
8000
6000
4000
2000
0
EDG5 +
vector
EDG5 +
Gq
EDG5 +
G16
EDG5 +
Gqi5

FIG 2E

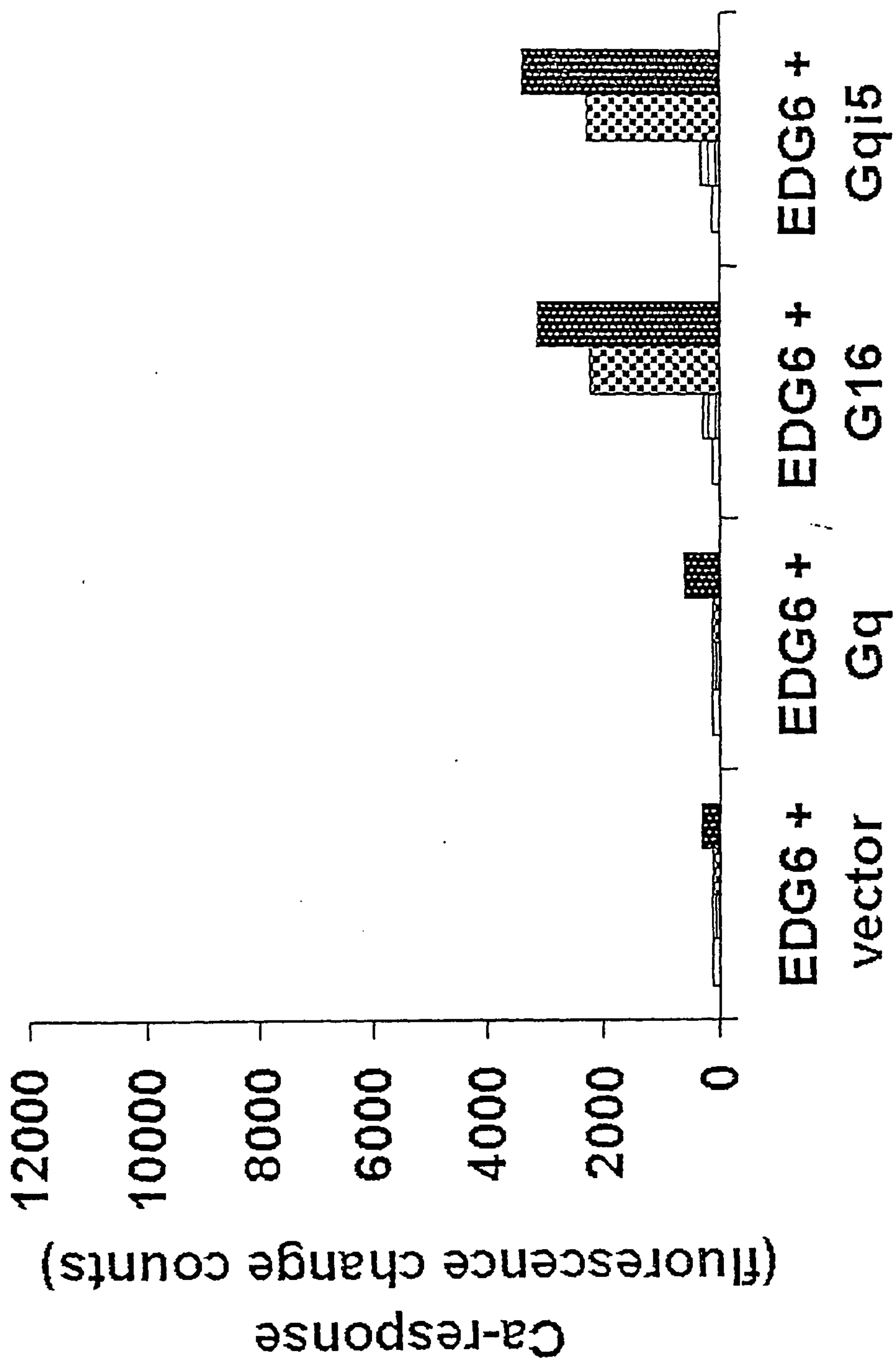

Ca-response
(fluorescence change counts)
12000
10000
8000
6000
4000
2000
0
EDG6 + vector
EDG6 + Gq
EDG6 + G16
EDG6 + Gqi5

FIG 2F

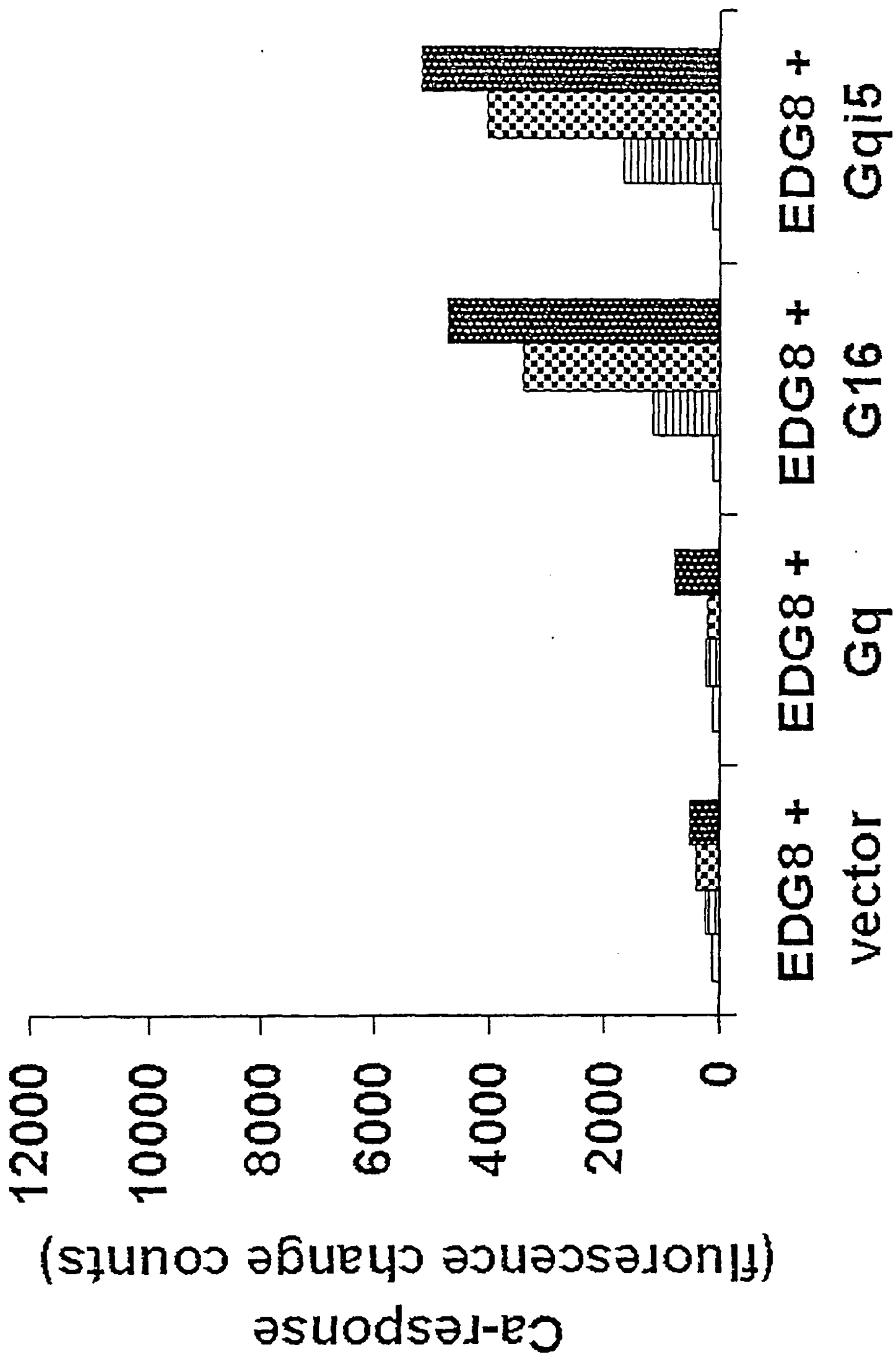
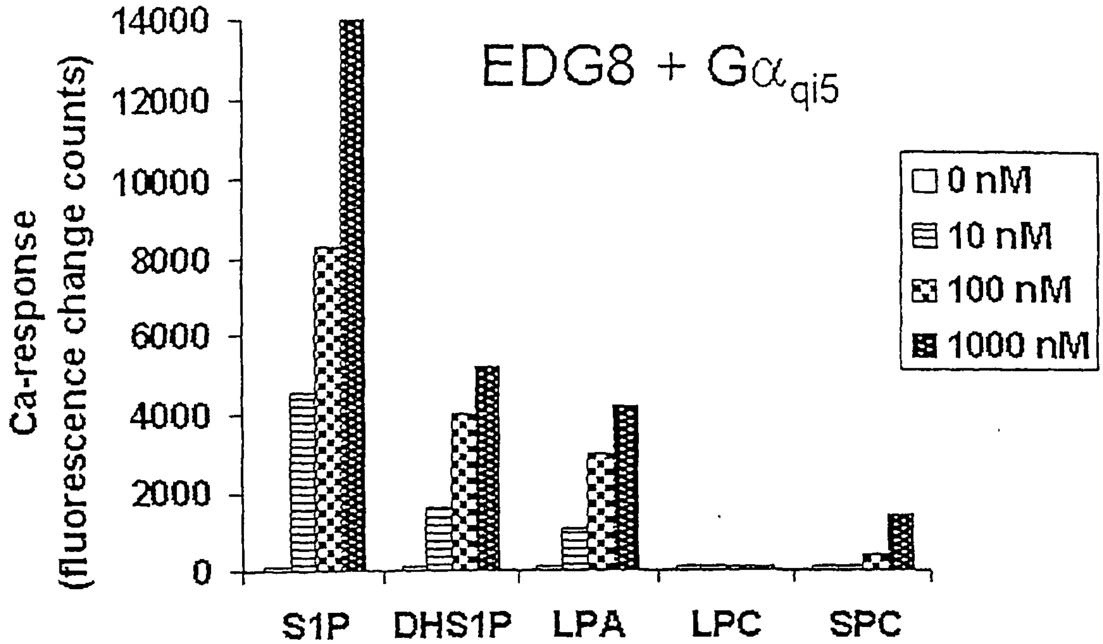

Ca-response
(fluorescence change counts)
12000
10000
8000
6000
4000
2000
0
EDG8 +
vector
EDG8 +
Gq
EDG8 +
G16
EDG8 +
Gqi5

FIG 3


EDG8 + $G\alpha_{qi5}$
Ca-response (fluorescence change counts)
14000
12000
10000
8000
6000
4000
2000
0
S1P
DHS1P
LPA
LPC
SPC
0 nM
10 nM
100 nM
1000 nM

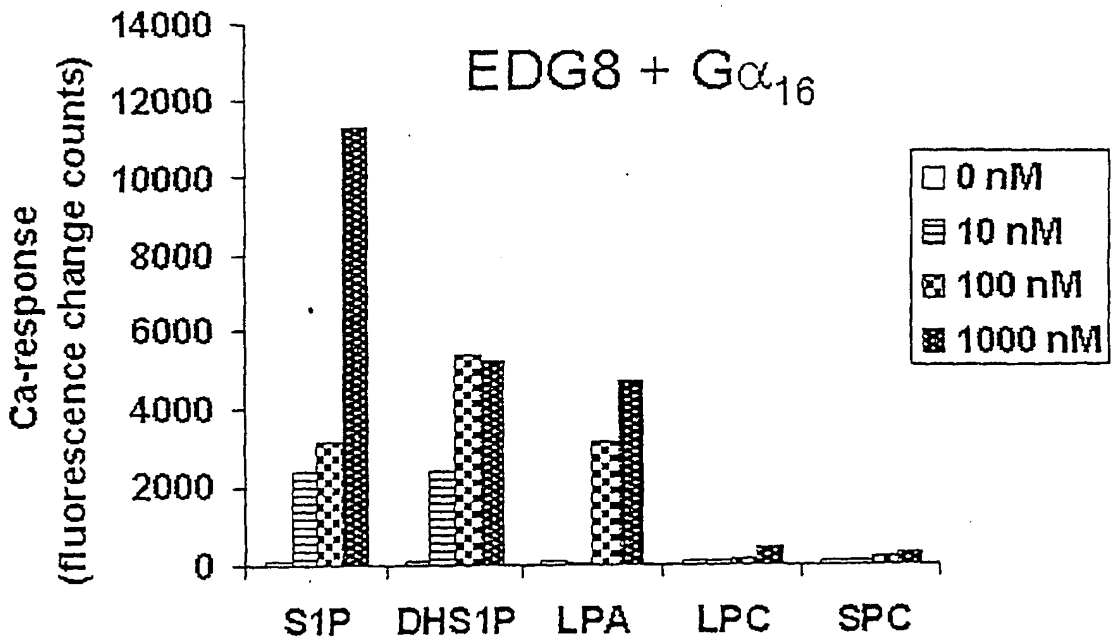

EDG8 + $G\alpha_{16}$
Ca-response (fluorescence change counts)
14000
12000
10000
8000
6000
4000
2000
0
S1P
DHS1P
LPA
LPC
SPC
0 nM
10 nM
100 nM
1000 nM

FIG 4

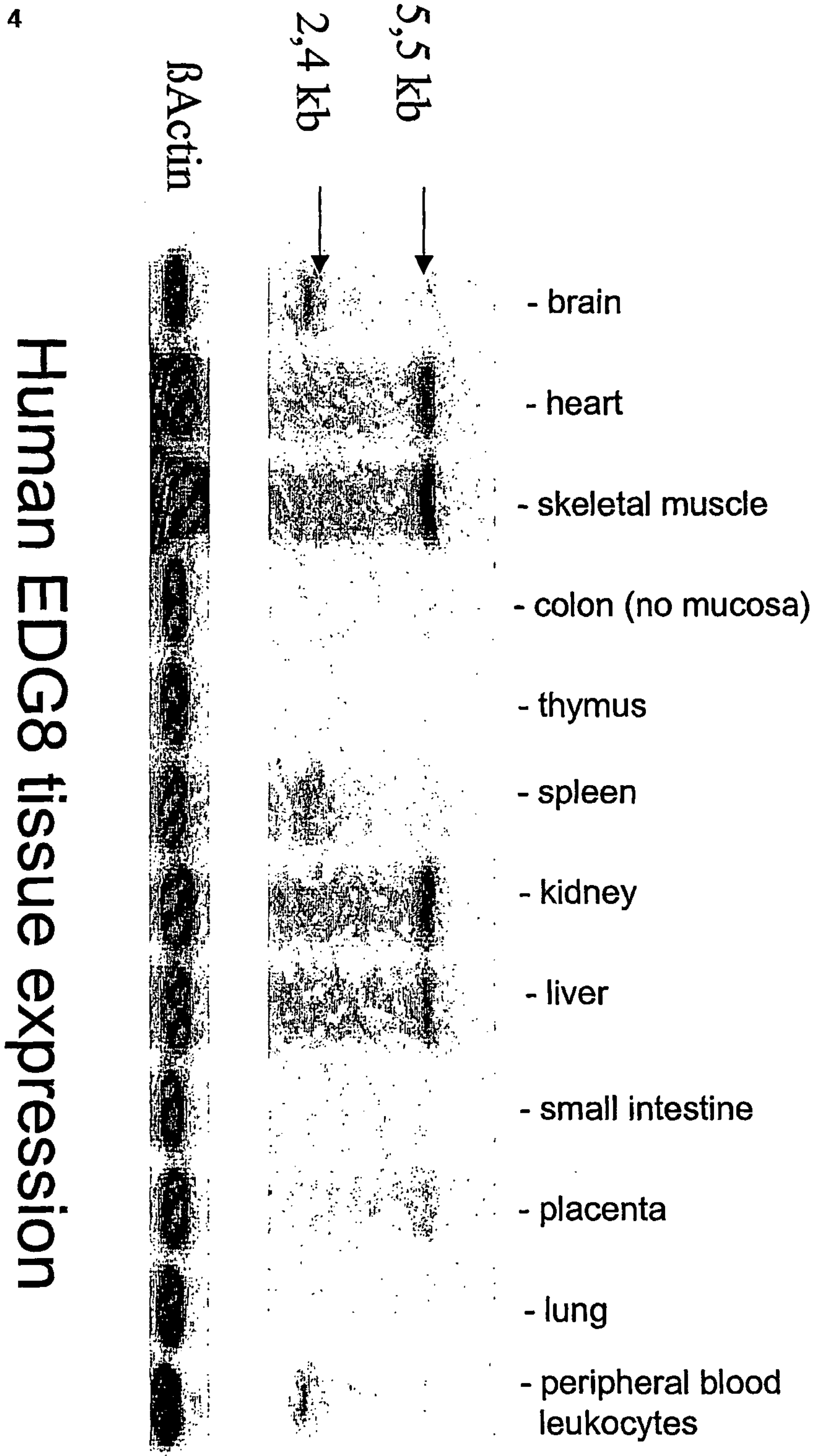

Human EDG8 tissue expression
βActin
2,4 kb
5,5 kb
- brain
- heart
- skeletal muscle
- colon (no mucosa)
- thymus
- spleen
- kidney
- liver
- small intestine
- placenta
- lung
- peripheral blood leukocytes

FIG. 5A

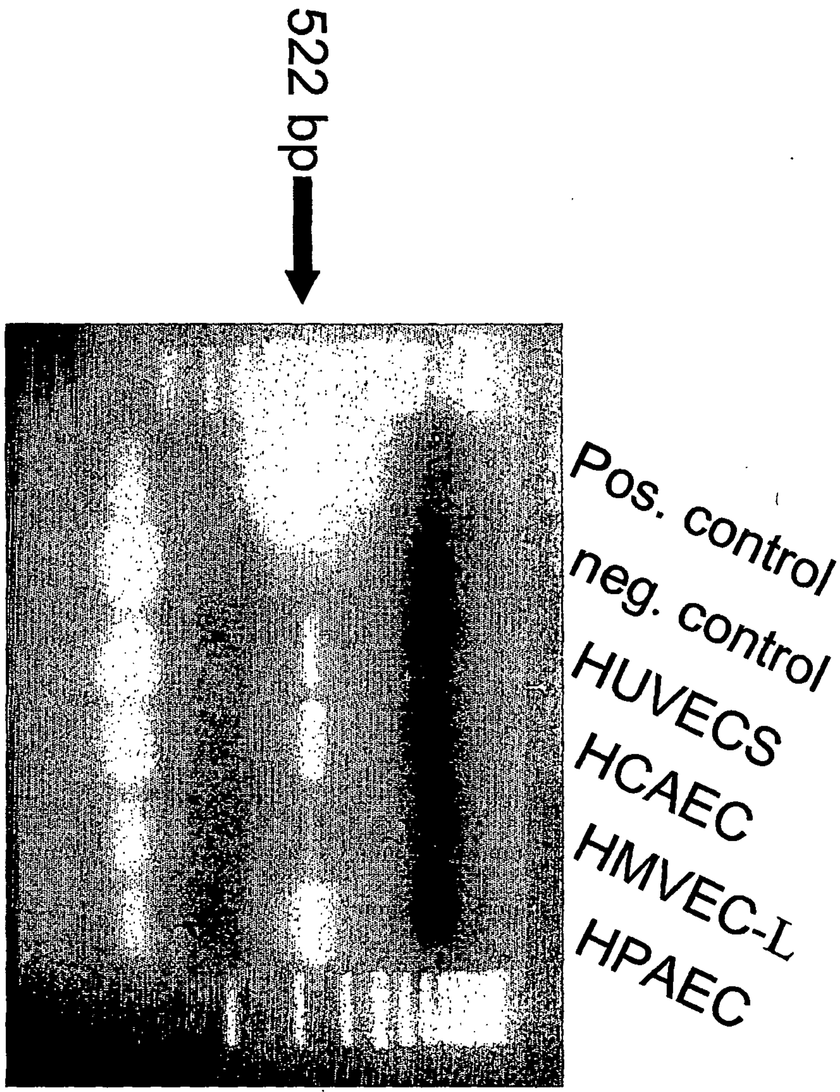


FIG 5B

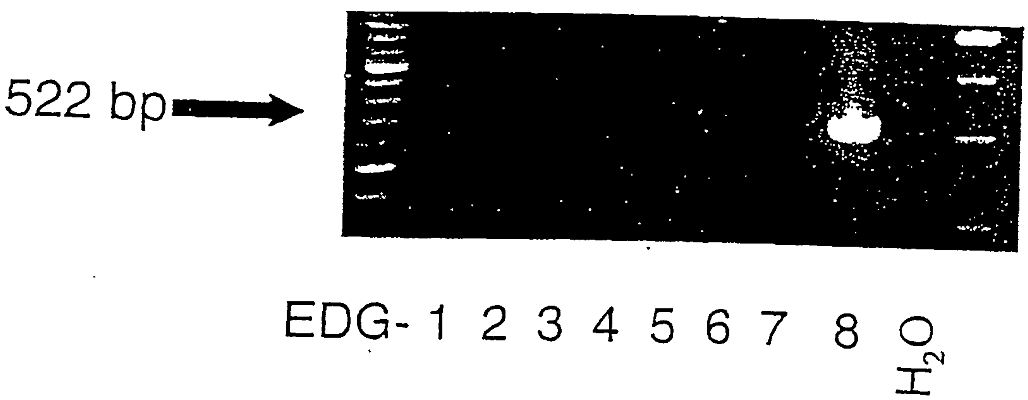


Fig. 6A

qi5 background

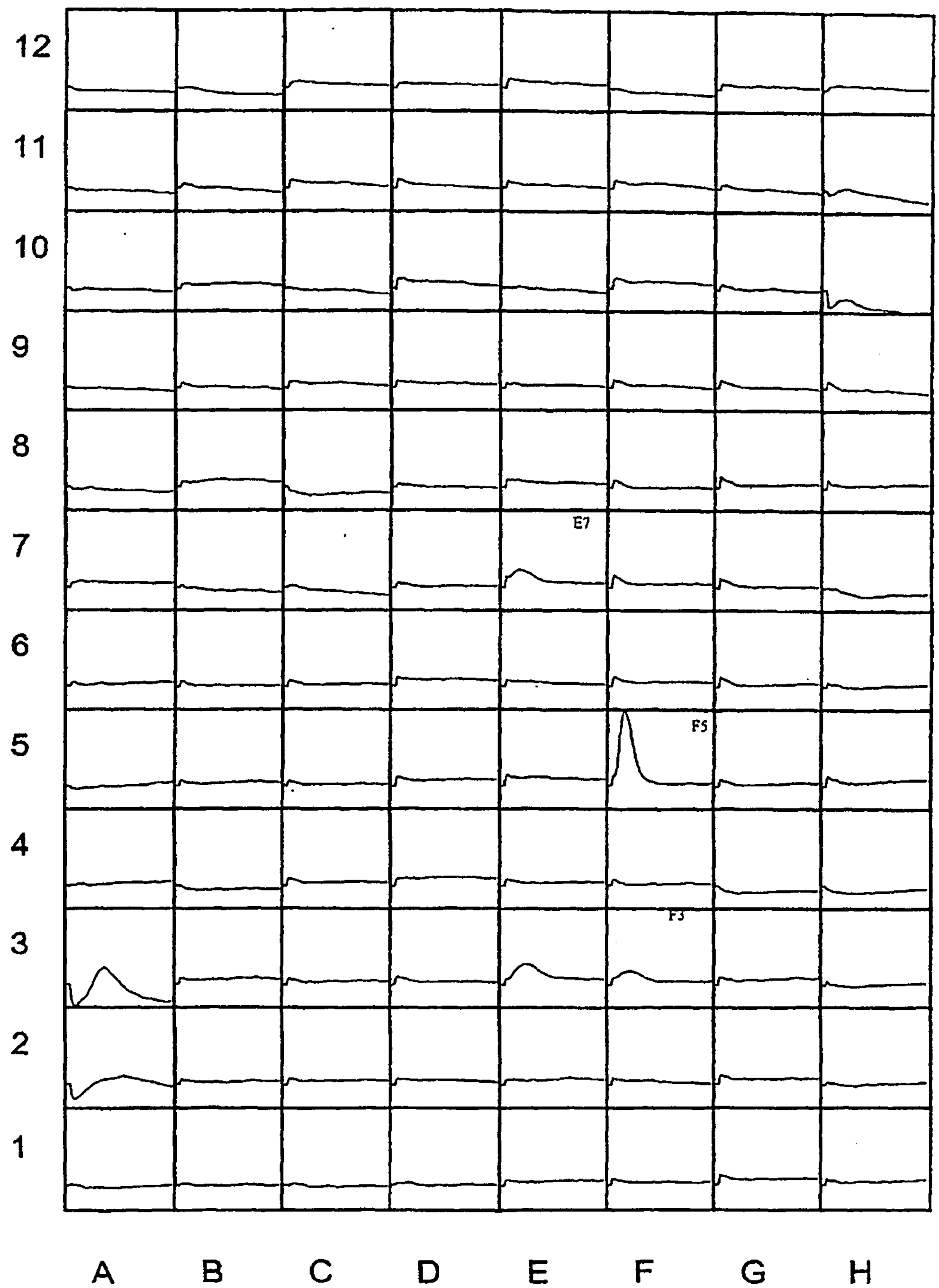


Fig. 6B

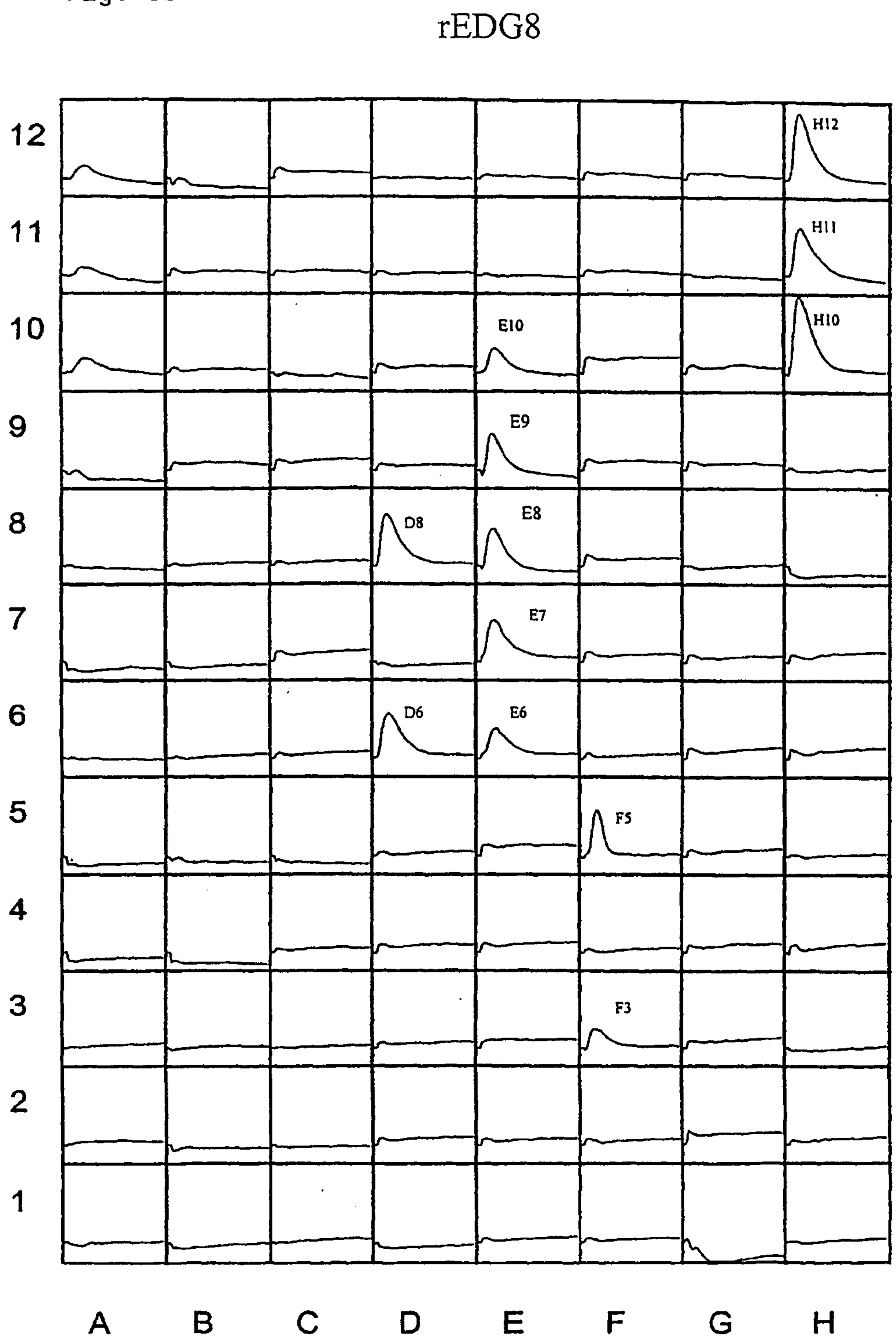

rEDG8
12
11
10
9
8
7
6
5
4
3
2
1
A
B
C
D
E
F
G
H
H12
H11
H10
E10
E9
E8
D8
E7
D6
E6
F5
F3

Fig.6C

# Fluorescence Change counts

| Wells | Lipid | background | rEDG8 | stand. response |
|---|---|---|---|---|
| H10-H12 | 1µM S1P | 0 | 5196 | 5196 |
| F5 | 1µM LPA | 5893 | 4327 | -1566 |
| F3 | 1µM cPAF | 1017 | 1570 | 553 |
| E10 | 1µM EPA PAF | 0 | 1354 | 1354 |
| E9 | 1µM AA PAF | 0 | 3121 | 3121 |
| E8 | 1µM Enantio PAF | 0 | 3883 | 3883 |
| E7 | 1µM paf C18:1 | 1256 | 3765 | 2509 |
| E6 | 1µM Lyso PAF | 0 | 2421 | 2421 |
| D8 | 1µM dhS1P | 0 | 5144 | 5144 |
| D6 | 1µM S1P | 0 | 3672 | 3672 |

Fig. 7A

qi5 background in HEK

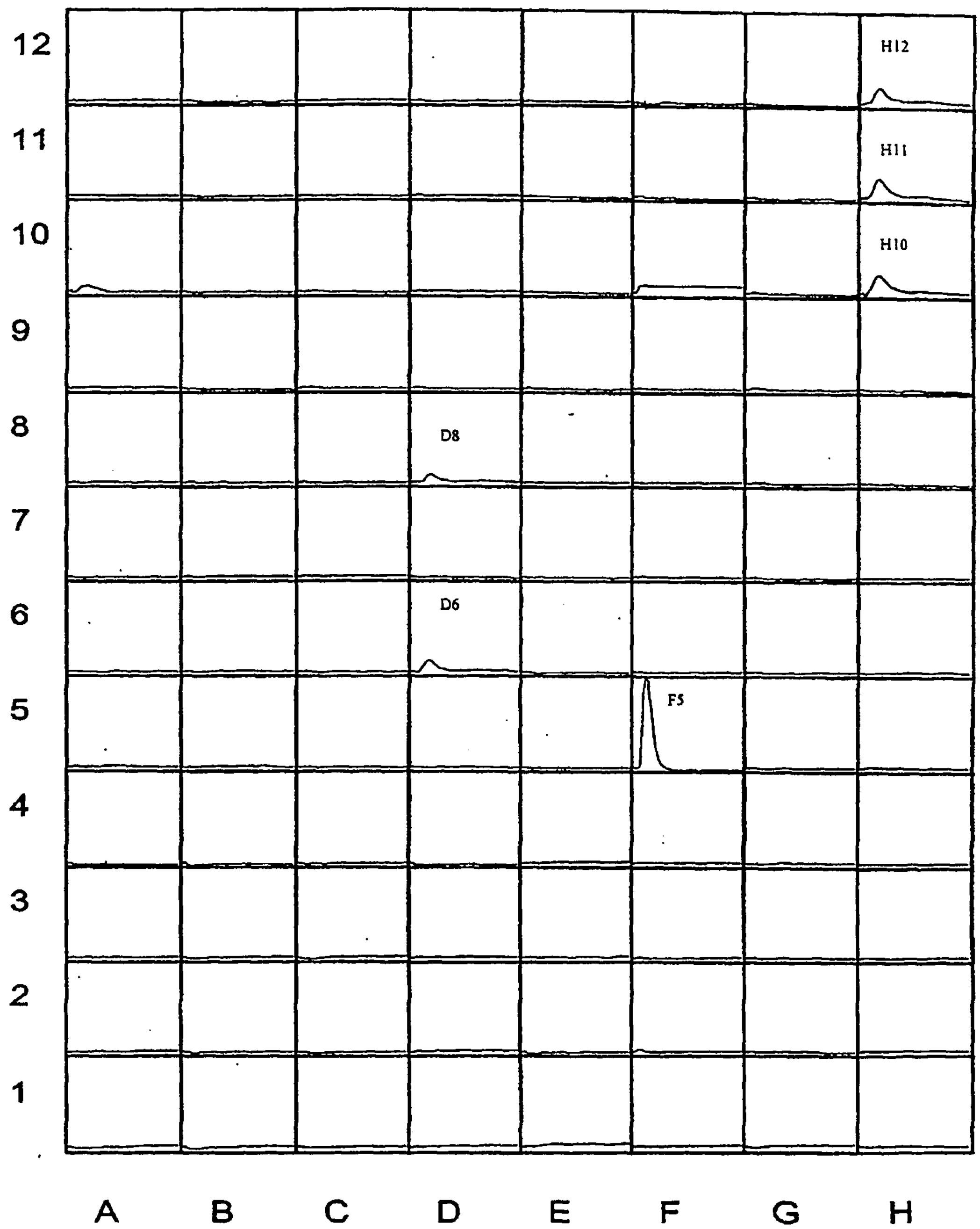
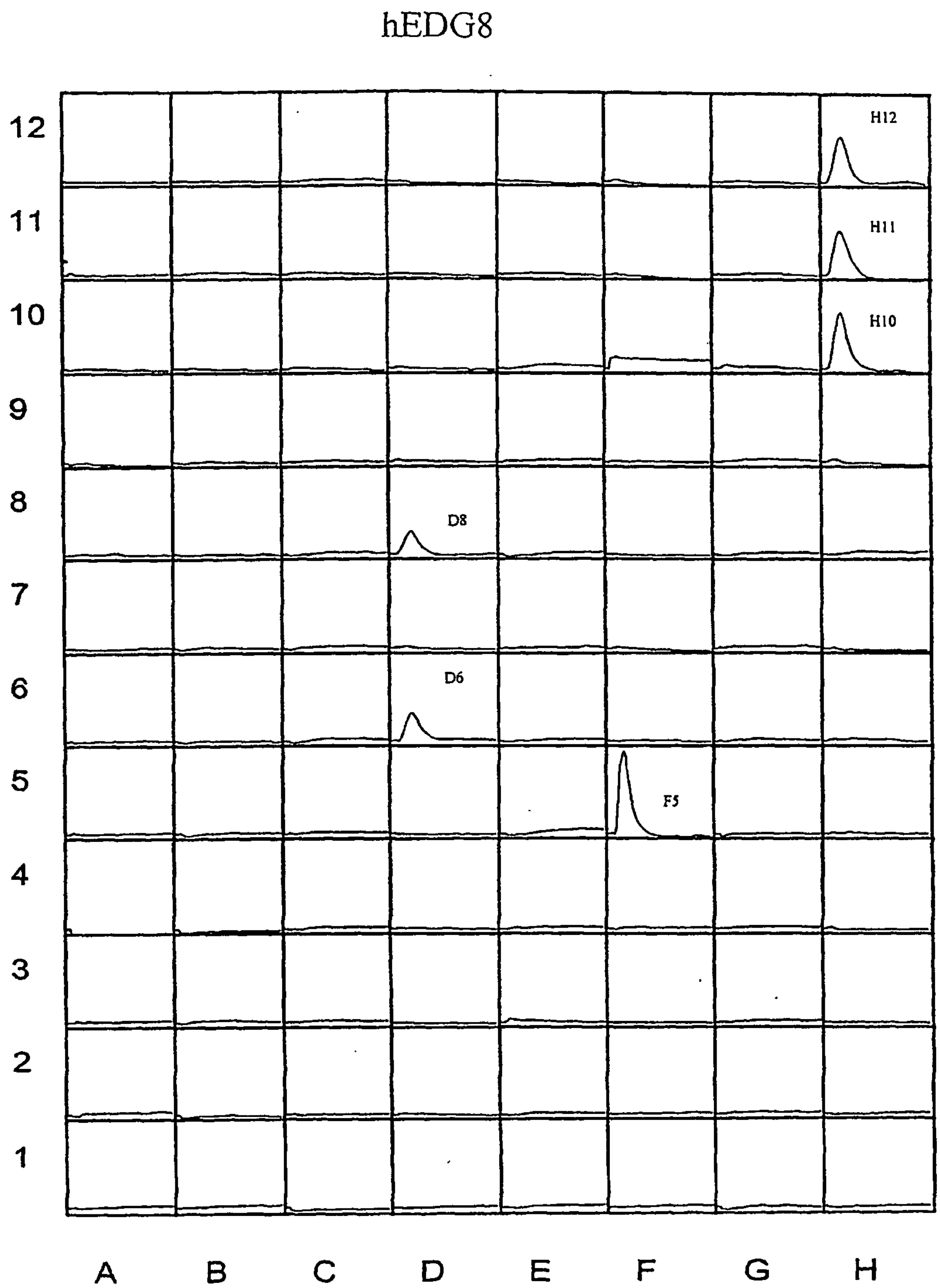


Fig. 7B



# Fluorescence change counts

| Wells | Lipid | background | hEDG8 | stand. response |
|---|---|---|---|---|
| H10-H12 | 1µM S1P | 3696 | 9493 | 5797 |
| F5 | 1µM LPA | 18004 | 16333 | -1671 |
| D8 | 1µM dhS1P | 1683 | 4522 | 2839 |
| D6 | 1µM S1P | 2273 | 5605 | 3332 |

Fig. 7C

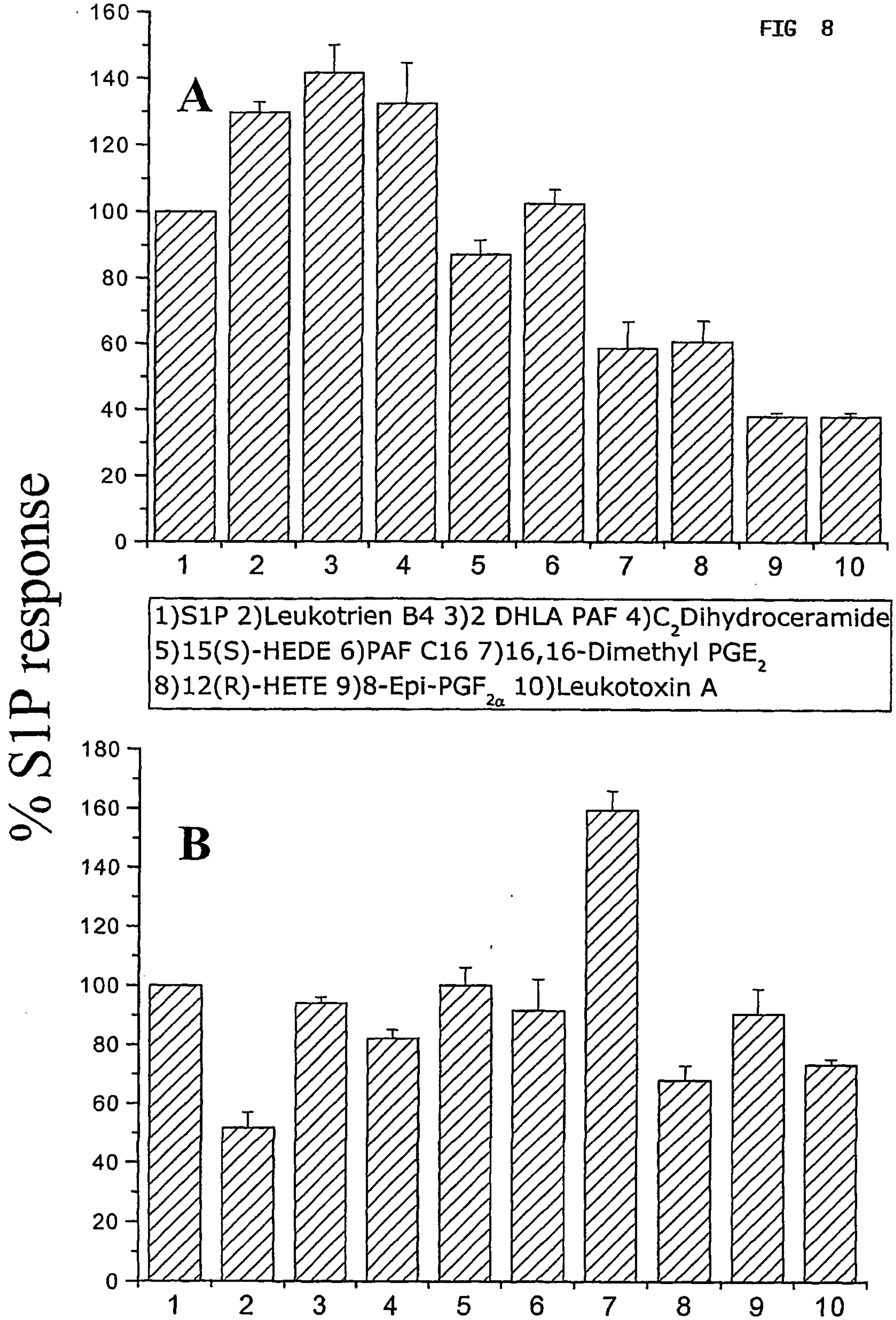

% S1P response
A
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
1)S1P 2)Leukotrien B4 3)2 DHLA PAF 4)$C_2$Dihydroceramide
5)15(S)-HEDE 6)PAF C16 7)16,16-Dimethyl $PGE_2$
8)12(R)-HETE 9)8-Epi-$PGF_{2\alpha}$ 10)Leukotoxin A
B
180
160
140
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10

FIG 8

FIG 9A

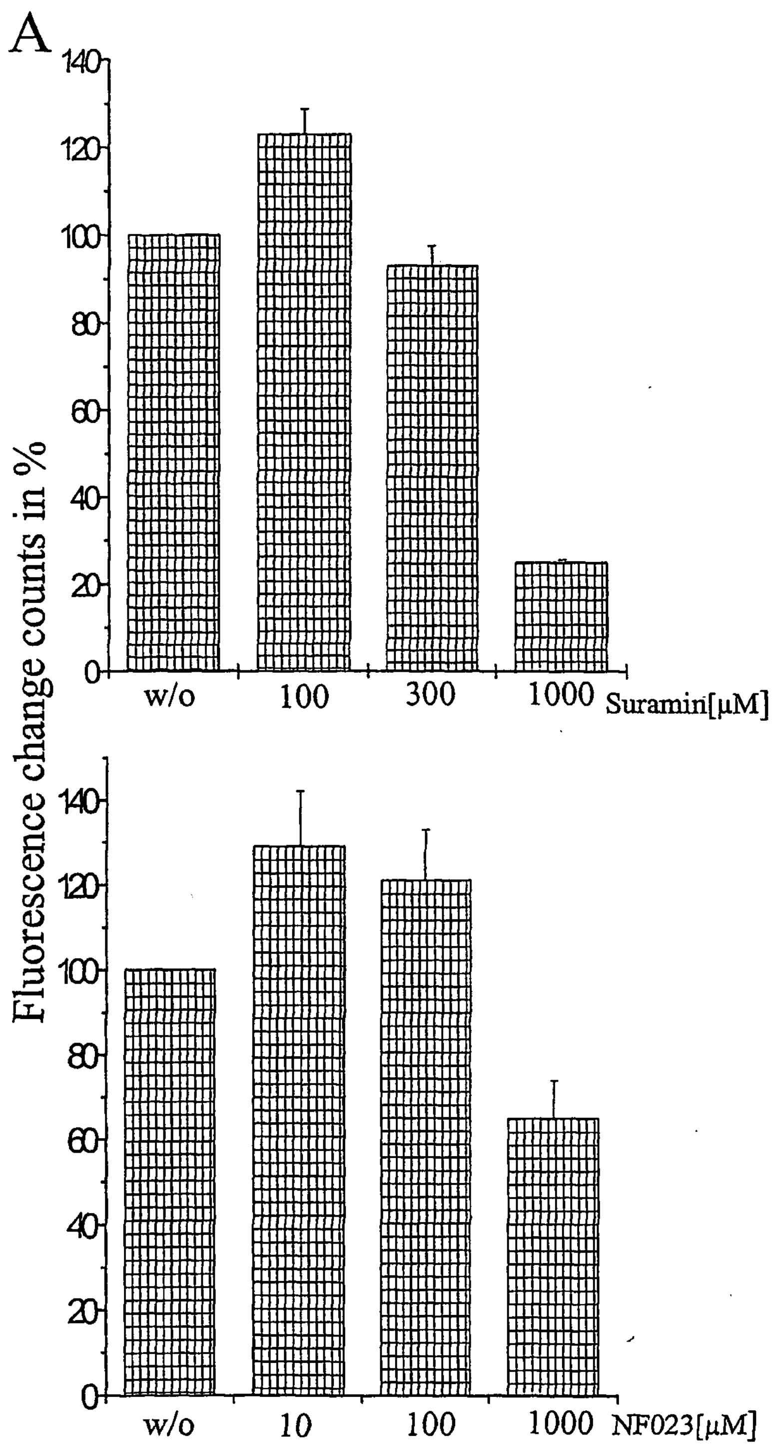
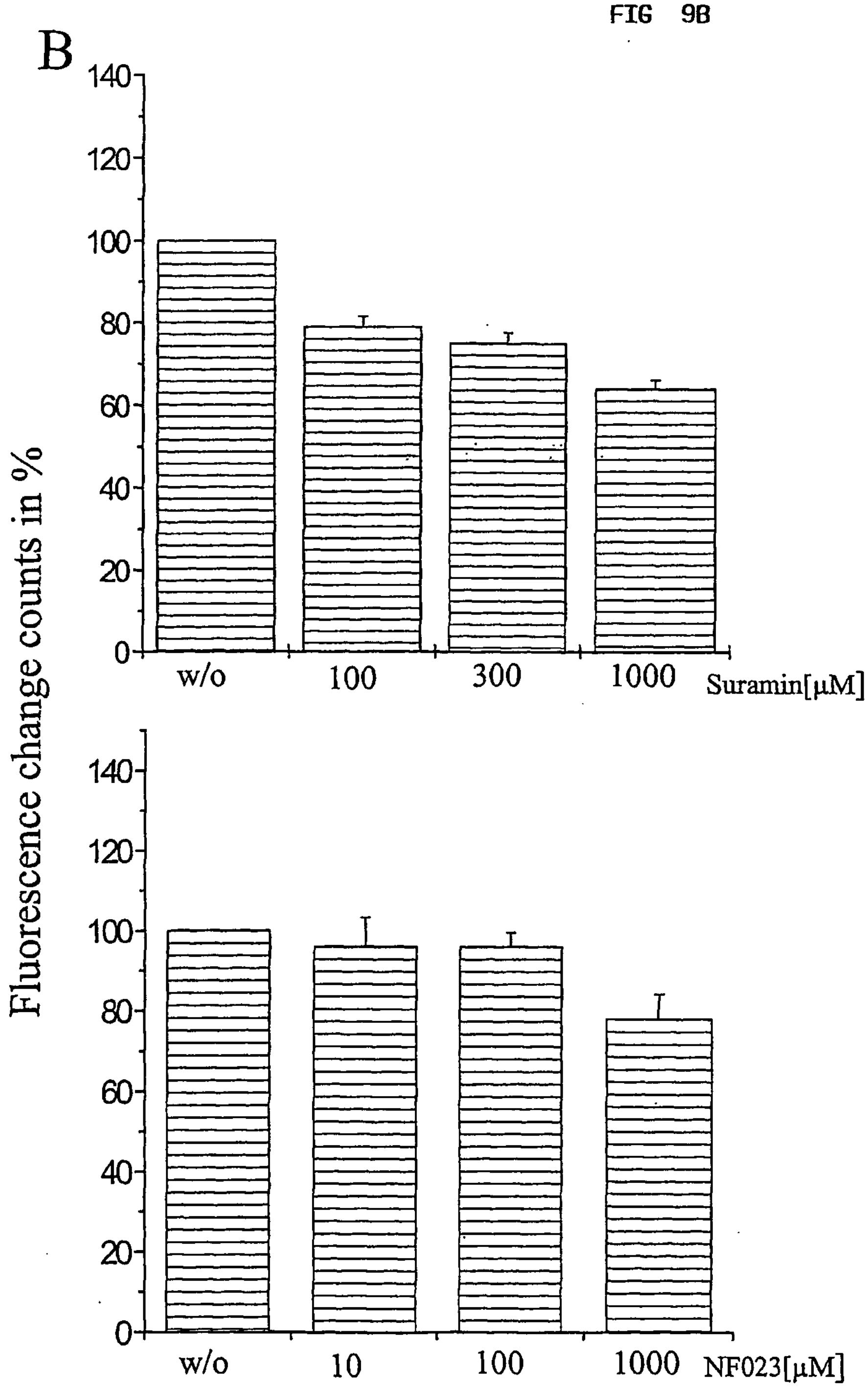

A
Fluorescence change counts in %
140
120
100
80
60
40
20
0
w/o
100
300
1000
Suramin[μM]
140
120
100
80
60
40
20
0
w/o
10
100
1000
NF023[μM]

FIG 9B


B
Fluorescence change counts in %
140
120
100
80
60
40
20
0
w/o
100
300
1000
Suramin[μM]
140
120
100
80
60
40
20
0
w/o
10
100
1000
NF023[μM]

SEQUENCE LISTING

<110> Aventis Pharma Deutschland GmbH

<120> EDG8 receptor, its preparation and use

<130> AVE D-2000/A024

<140>
<141>

<150> 00108858.2
<151> 2000-04-26

<150> 00116589.3
<151> 2000-08-01

<160> 2

<170> PatentIn Ver. 2.1

<210> 1
<211> 1197
<212> DNA
<213> Homo sapiens

<400> 1
atggagtcgg ggctgctgcg gccggcgccg gtgagcgagg tcatcgtcct gcattacaac 60
tacaccggca agctccgcgg tgcgcgctac cagccgggtg ccggcctgcg cgccgacgcc 120
gtggtgtgcc tggcggtgtg cgccttcatc gtgctagaga atctagccgt gttgttggtg 180
ctcggacgcc acccgcgctt ccacgctccc atgttcctgc tcctgggcag cctcacgttg 240
tcggatctgc tggcaggcgc cgcctacgcc gccaacatcc tactgtcggg gccgctcacg 300
ctgaaactgt cccccgcgct ctggttcgca cgggagggag gcgtcttcgt ggcactcact 360
gcgtccgtgc tgagcctcct ggccatcgcg ctggagcgca gcctcaccat ggcgcgcagg 420
gggcccgcgc ccgtctccag tcgggggcgc acgctggcga tggcagccgc ggcctggggc 480
gtgtcgctgc tcctcgggct cctgccagcg ctgggctgga attgcctggg tcgcctggac 540
gcttgctcca ctgtcttgcc gctctacgcc aaggcctacg tgctcttctg cgtgctcgcc 600
ttcgtgggca tcctggccgc tatctgtgca ctctacgcgc gcatctactg ccaggtacgc 660
gccaacgcgc ggcgcctgcc ggcacggccc gggactgcgg ggaccacctc gacccgggcg 720
cgtcgcaagc cgcgctcgct ggccttgctg cgcacgctca gcgtggtgct cctggccttt 780
gtggcatgtt ggggcccccct cttcctgctg ctgttgctcg acgtggcgtg cccggcgcgc 840
acctgtcctg tactcctgca ggccgatccc ttcctgggac tggccatggc caactcactt 900
ctgaacccca tcatctacac gctcaccaac cgcgacctgc gccacgcgct cctgcgcctg 960
gtctgctgcg gacgccactc ctgcggcaga gacccgagtg gctcccagca gtcggcgagc 1020
gcggctgagg cttccggggg cctgcgccgc tgcctgcccc cgggccttga tgggagcttc 1080
agcggctcgg agcgctcatc gccccagcgc gacgggctgg acaccagcgg ctccacaggc 1140
agccccggtg cacccacagc cgcccggact ctggtatcag aaccggctgc agactga 1197

<210> 2
<211> 398
<212> PRT
<213> Homo sapiens

<400> 2
Met Glu Ser Gly Leu Leu Arg Pro Ala Pro Val Ser Glu Val Ile Val
1               5                   10                  15

Leu His Tyr Asn Tyr Thr Gly Lys Leu Arg Gly Ala Arg Tyr Gln Pro
            20                  25                  30

Gly Ala Gly Leu Arg Ala Asp Ala Val Val Cys Leu Ala Val Cys Ala
        35                  40                  45

Phe Ile Val Leu Glu Asn Leu Ala Val Leu Leu Val Leu Gly Arg His
    50                  55                  60

Pro Arg Phe His Ala Pro Met Phe Leu Leu Leu Gly Ser Leu Thr Leu
65                  70                  75                  80

Ser Asp Leu Leu Ala Gly Ala Ala Tyr Ala Ala Asn Ile Leu Leu Ser
                85                  90                  95

Gly Pro Leu Thr Leu Lys Leu Ser Pro Ala Leu Trp Phe Ala Arg Glu
            100                 105                 110

Gly Gly Val Phe Val Ala Leu Thr Ala Ser Val Leu Ser Leu Leu Ala
        115                 120                 125

Ile Ala Leu Glu Arg Ser Leu Thr Met Ala Arg Arg Gly Pro Ala Pro
    130                 135                 140

Val Ser Ser Arg Gly Arg Thr Leu Ala Met Ala Ala Ala Ala Trp Gly
145                 150                 155                 160

Val Ser Leu Leu Leu Gly Leu Leu Pro Ala Leu Gly Trp Asn Cys Leu
                165                 170                 175

Gly Arg Leu Asp Ala Cys Ser Thr Val Leu Pro Leu Tyr Ala Lys Ala
            180                 185                 190

Tyr Val Leu Phe Cys Val Leu Ala Phe Val Gly Ile Leu Ala Ala Ile
        195                 200                 205

Cys Ala Leu Tyr Ala Arg Ile Tyr Cys Gln Val Arg Ala Asn Ala Arg
    210                 215                 220

Arg Leu Pro Ala Arg Pro Gly Thr Ala Gly Thr Thr Ser Thr Arg Ala
225 230 235 240

Arg Arg Lys Pro Arg Ser Leu Ala Leu Leu Arg Thr Leu Ser Val Val
245 250 255

Leu Leu Ala Phe Val Ala Cys Trp Gly Pro Leu Phe Leu Leu Leu Leu
260 265 270

Leu Asp Val Ala Cys Pro Ala Arg Thr Cys Pro Val Leu Leu Gln Ala
275 280 285

Asp Pro Phe Leu Gly Leu Ala Met Ala Asn Ser Leu Leu Asn Pro Ile
290 295 300

Ile Tyr Thr Leu Thr Asn Arg Asp Leu Arg His Ala Leu Leu Arg Leu
305 310 315 320

Val Cys Cys Gly Arg His Ser Cys Gly Arg Asp Pro Ser Gly Ser Gln
325 330 335

Gln Ser Ala Ser Ala Ala Glu Ala Ser Gly Gly Leu Arg Arg Cys Leu
340 345 350

Pro Pro Gly Leu Asp Gly Ser Phe Ser Gly Ser Glu Arg Ser Ser Pro
355 360 365

Gln Arg Asp Gly Leu Asp Thr Ser Gly Ser Thr Gly Ser Pro Gly Ala
370 375 380

Pro Thr Ala Ala Arg Thr Leu Val Ser Glu Pro Ala Ala Asp
385 390 395

# INTERNATIONAL SEARCH REPORT

International Application No
. . ./EP 01/04283

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 C12N15/12 C07K14/705 C12Q1/68 G01N33/52 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, STRAND, EMBL, BIOSIS, MEDLINE, EMBASE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 00 11166 A (MILLENNIUM PHARM INC) 2 March 2000 (2000-03-02) Note: 99.8% nt seq identity of SEQ ID NO:2 with SEQ ID NO:1 in 1197 bp overlap, 99.8% aa seq identity of SEQ ID NO:1 with SEQ ID NO:2 in 398 aa overlap. the whole document page 15, line 13 -page 16, line 29 page 27, line 10 -page 31, line 19 page 47, line 25 -page 48, line 14 --- | 1-30 |

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 5 September 2001 | 26/09/2001 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | van de Kamp, M |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | DATABASE EMBL 'Online! EBI; ID AC011461, AC AC011461, 8 October 1999 (1999-10-08) "Homo sapiens chromosome 19 clone CTC-429L19, WORKING DRAFT SEQUENCE, 4 ordered pieces" XP002176772 Note: 100.0% nt seq identity with SEQ ID NO:1 in 1197 nt overlap (41760-42956:1-1197), 100.0% aa seq identity of translated nts with SEQ ID NO:2 in 398 aa overlap. page 1 page 12 | 1-6 |
| X | WO 00 22129 A (ARENA PHARMACEUTICALS INC ;LIAW CHEN W (US); BEHAN DOMINIC P (US);) 20 April 2000 (2000-04-20) Note: 99.7 % nt seq identity of SEQ ID NO:31 with SEQ ID NO:1 in 1197 bp overlap, 99.7 % aa seq identity of SEQ ID NO:32 with SEQ ID NO:2 in 398 aa overlap. page 32, line 9-21 page 28-69; examples 1-3 | 1-21, 27-30 |
| A | GLICKMAN M ET AL.: "Molecular cloning, tissue-specific expression, and chromosomal localization of a novel nerve growth factor-regulated G-protein-coupled receptor, nrg-1." MOL. CELL. NEUROSCI., vol. 14, no. 2, August 1999 (1999-08), pages 141-152, XP000939383 cited in the application abstract page 142, right-hand column, line 43 -page 144, left-hand column, line 20 | 1-30 |
| A | WO 99 19513 A (LXR BIOTECHNOLOGY INC ;ERIKSON JAMES (US); KIEFER MICHAEL (US); GO) 22 April 1999 (1999-04-22) page 29, line 1 -page 33, line 15 | 1-30 |
| A | AN S ET AL.: "Signaling mechanisms and molecular characteristics of G-protein-coupled receptors for lysophosphatidic acid and sphingosine 1-phosphate" JOURNAL OF CELLULAR BIOCHEMISTRY - SUPPLEMENT, vol. 30/31, December 1998 (1998-12), pages 147-157, XP002127866 the whole document | 1-30 |

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | HLA T ET AL.: "Sphingosine-1-phosphate signalling via the EDG-1 family of G-protein-coupled receptors"<br>ANN N Y ACAD SCI,<br>vol. 905, April 2000 (2000-04), pages 16-24, XP000939014<br>the whole document<br>--- | 1-30 |
| P,X | WO 01 04139 A (MICHALOVICH DAVID ;SMITHKLINE BEECHAM PLC (GB); ELSHOURBAGY NABIL) 18 January 2001 (2001-01-18)<br>Note: 100.0% aa seq identity of SEQ ID NO:2 in 398 aa overlap<br>the whole document<br>page 7, line 27 -page 8, line 26<br>examples 1-9<br>claims 1-16<br>--- | 1-30 |
| P,X | EP 1 090 925 A (PFIZER LTD ;PFIZER (US)) 11 April 2001 (2001-04-11)<br>Note: 100.0% nt seq identity of SEQ ID NO:1 with SEQ ID NO:1 in 1195 nt overlap (1-1195:1-1195), 100.0% aa seq identity of SEQ ID NO:2 with SEQ ID NO:2 in 398 aa overlap<br>the whole document<br>page 34, line 5 -page 35, line 25<br>page 36, line 1 -page 37, line 21<br>--- | 1-30 |
| P,X | DATABASE EM_HUM 'Online!<br>EMBL;<br>ID AF317676, AC AF317676,<br>6 December 2000 (2000-12-06)<br>IM D ET AL.: "Homo sapiens sphingosine 1-phosphate receptor Edg-8 gene, complete cds"<br>XP002176773<br>Note: 99.9% nt seq identity in 1197 nt overlap, 100.0% aa seq identity in 398 aa overlap<br>the whole document<br>--- | 1-7,<br>15-17 |

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | IM D-S ET AL.: "Characterization of a novel sphingosine 1-phosphate receptor, Edg-8"<br>J. BIOL. CHEM.,<br>vol. 275, no. 19,<br>12 May 2000 (2000-05-12), pages 14281-14286, XP000939039<br>Note: 80.7 % nt seq identity with SEQ ID NO:1 in 1046 bp overlap, 86.8 % aa seq identity with SEQ ID NO:2 in 401 aa overlap.<br>abstract<br>page 14283, left-hand column, line 2-8<br>page 14283, right-hand column, line 1<br>-page 14285, left-hand column, line 20<br>page 14286, left-hand column, line 27-39<br>----- | 1-7,<br>15-17 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/04283

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| WO 0011166 | A | 02-03-2000 | AU | 5575299 A | 14-03-2000 |
| | | | EP | 1105480 A | 13-06-2001 |
| WO 0022129 | A | 20-04-2000 | AU | 6299199 A | 01-05-2000 |
| | | | AU | 6430799 A | 01-05-2000 |
| | | | EP | 1121431 A | 08-08-2001 |
| | | | WO | 0021987 A | 20-04-2000 |
| | | | WO | 0022131 A | 20-04-2000 |
| WO 9919513 | A | 22-04-1999 | AU | 9794598 A | 03-05-1999 |
| WO 0104139 | A | 18-01-2001 | NONE | | |
| EP 1090925 | A | 11-04-2001 | JP | 2001161382 A | 19-06-2001 |